Exhibit 1

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Masoprocol Lowers Blood Pressure in Rats With Fructose-Induced Hypertension

Maya S. Gowri, Gerald M. Reaven, and Salman Azhar

Rats with fructose-induced hypertension were treated by oral gavage with either masoprocol (nordihydroguaiaretic acid) or vehicle. Masoprocol treatment resulted in significantly ($P < .05$ to .001) lower values for systolic blood pressure (120 ± 3 *v* 164 ± 5 mm Hg), as well as plasma insulin (30 ± 5 *v* 44 ± 4 μU/mL), free fatty acid (551 ± 20 *v* 692 ± 22 μEq/L), and triglyceride (79 ± 5 *v* 219 ± 32 mg/dL) concentrations. These results indicate that masoprocol, a lipoxygenase inhibitor, is able to lower blood pressure, as well as improve the metabolic abnormalities present in a rodent model of hypertension that simulates the characteristic of many patients with essential hypertension. Am J Hypertens 1999;12:744–746 © 1999 American Journal of Hypertension, Ltd.

KEY WORDS: Fructose-induced hypertension, masoprocol, lipoxygenase inhibitors.

Recently, we have shown that oral administration of masoprocol (nordihydroguaiaretic acid) lowers plasma glucose and triglyceride (TG) concentrations in rodent models of Type 2 diabetes,[1,2] as well as preventing hypertriglyceridemia in nondiabetic rats fed a fructose-enriched diet.[3] Masoprocol is a well-known lipoxygenase inhibitor,[4] and we are unaware of any published evidence that compounds of this chemical class have beneficial effects in rats with either hyperglycemia or hypertriglyceridemia. However, evidence has been published that inhibitors of 12-lipoxygenase activity can lower blood pressure in spontaneously hypertensive rats, as well as in rats with renovascular hypertension.[5,6] Because hypertension also develops in fructose-fed rats,[7,8] we initiated the current study aimed at evaluating the effect of masoprocol in this form of rodent hypertension.

METHODS

Male Sprague-Dawley rats (Harlan Sprague Dawley, Indianapolis, IN), initially weighing 175 to 199 g were used for all experiments. Before dietary manipulation, all rats were fed Purina Rat Chow (no. 5012; St. Louis, MO) and water ad libitum and maintained on a 12-h (06:00 to 18:00) light–dark cycle. The rats were then placed on a diet (TD 78463; Harlan Teklad, Madison, WI) that provided 60% of their total calories as fructose.[7,8] The fructose-enriched diet was given for 11 days, during which time the rats were acclimated to the procedure of blood pressure measurement.

At the end of this initial dietary period, blood pressure was determined, and the rats were randomly divided into two groups. Both groups were maintained on the fructose-enriched diet, but one group was gavaged with masoprocol (80 mg/kg, twice daily), dissolved in gelucire, whereas the other was treated in the same manner with vehicle alone.

Blood pressure was measured before and 4 days after treatment with either masoprocol or vehicle. In both instances, the general procedure was similar.

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Rats were removed from the animal room and taken to the laboratory at 09:00. They were allowed free access to water and were kept in a quiet area before the blood pressure was measured at 13:00. The tail-cuff method, without external preheating, was used to measure the systolic blood pressure.[9] Ambient temperature was kept at 30°C. The equipment used included magnetic animal holders connected with manual scanner (model 65-12, IITC, Inc., Woodland Hills, CA), pulse amplifier (model 59, IITC, Inc.), and dual-channel recorder (model 1202, Linear Instrs. Corp., Reno, NV). The systolic blood pressure was measured in the conscious state and has been shown with this technique to be similar to that obtained by direct arterial cannulation.[10] The mean of consecutive readings was used as the measurement of the systolic blood pressure of each rat for that day. The final blood pressure determinations were performed on the afternoon after the last morning dose of masoprocol or vehicle.

In approximately half of the rats studied, tail vein blood was removed at 13:00 (4 h after removal of food), centrifuged, frozen, and later assayed for plasma glucose,[11] insulin,[12] and triglyceride[13] concentrations. Plasma free fatty acid concentration was assayed enzymatically by the ACS-ACOD method using a commercial kit (Waro Chemicals Inc., Richmond, VA).

Results are expressed as mean ± SEM, and statistical significance of differences between the two groups compared by one-way analysis of variance.

RESULTS

Both groups of rats tolerated the intervention without any obvious problems, and their weight was essentially identical at the end of the treatment period, 258 ± 4 g *v* 250 ± 13 g for the vehicle and masoprocol-treated groups, respectively.

Blood pressure values, before and after treatment, are shown in Figure 1. It is apparent that although the baseline blood pressures were similar in the two groups, they diverged dramatically once the intervention began. Blood pressure continued to increase in vehicle-treated rats ($P < .01$), whereas blood pressure actually decreased below baseline ($P < .001$) in rats treated with masoprocol. Consequently, the blood pressure averaged 44 mm Hg lower in the masoprocol-treated rats as compared to the control rats ($P < .0001$) by the end of the study.

Plasma glucose, insulin, free fatty acid, and triglyceride concentrations after treatment with either masoprocol or vehicle are shown in Table 1. Although plasma glucose concentrations were similar in the two groups, masoprocol-treated rats had significantly lower plasma insulin, free fatty acid, and triglyceride concentrations.

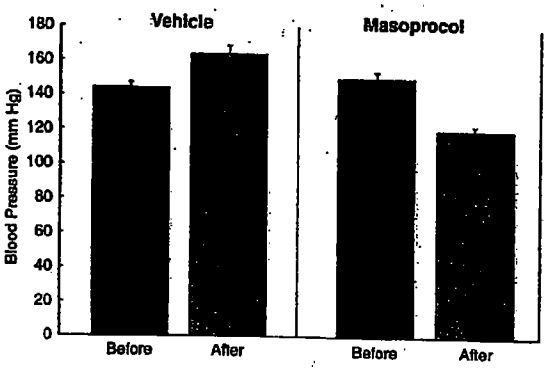

FIGURE 1. *Blood pressure before and after treatment with vehicle or masoprocol. Blood pressure decreased significantly in masoprocol-treated rats* ($P < .001$).

DISCUSSION

Previous results have shown that the oral administration of phenidone, a lipoxygenase inhibitor, lowers blood pressure in rats with renovascular or spontaneous hypertension.[5,6] In the present study we have shown that the ability of lipoxygenase inhibitors to lower blood pressure is not limited to rats with genetic or spontaneous forms of hypertension, or to a specific lipoxygenase inhibitor. Demonstration that the lipoxygenase inhibitor masoprocol can lower blood pressure in a form of dietary-induced hypertension, in an outbred rat strain, significantly expands that possible role played by the lipoxygenase pathway in the regulation of blood pressure.

In addition to hypertension, fructose-fed rats are also relatively insulin resistant, hyperinsulinemic, and hypertriglyceridemic.[7,8] It is obvious from the results in Table 1 that, in addition to lowering blood pressure, masoprocol treatment was able to improve all of the untoward manifestations associated with consumption of fructose-enriched diets.

Although the results of this study are straightforward, they leave many questions unanswered. First, previous studies evaluating the antihypertensive effect of lipoxygenase inhibitors on the blood pressure

TABLE 1. PLASMA GLUCOSE, INSULIN, FREE FATTY ACID, AND TRIGLYCERIDE CONCENTRATIONS (N = 12)

Variable	Vehicle	Masoprocol	*P*
Glucose (mg/dL)	135 ± 6	140 ± 7	NS
Insulin (μU/mL)	44 ± 4	30 ± 5	<.05
Free fatty acid (μEq/L)	692 ± 30	551 ± 20	<.05
Triglyceride (mg/dL)	219 ± 32	79 ± 5	<.001

of rats with spontaneous or renovascular hypertension[5,6] were carried out over several weeks, whereas blood pressure of fructose-fed rats decreased within 1 week of masoprocol treatment. Whether this effect could be maintained over longer periods of time remains to be seen.

Second, the mechanistic explanation for the hemodynamic and metabolic effects of masoprocol documented in this study are not self-evident. As indicated above, fructose-fed rats are insulin resistant, hyperinsulinemic, and hypertriglyceridemic.[7,8] Furthermore, we have shown that all of these abnormalities will improve by manipulations that enhance insulin sensitivity or that alleviate hyperinsulinemia.[14,15] Thus, it could be speculated that masoprocol increases insulin sensitivity in fructose-fed rats, thereby leading to a concomitant decrease in blood pressure and plasma insulin and triglyceride concentrations. This possibility is supported by evidence that masoprocol enhances insulin sensitivity in rodent models of Type 2 diabetes.[1,2] If the hemodynamic and metabolic effects of masoprocol in fructose-fed rats are secondary to its ability to enhance insulin-mediated glucose disposal, this may be related to an effect on magnesium metabolism, given evidence that magnesium deficiency can produce insulin resistance[16] and magnesium supplementation inhibits fructose-induced insulin resistance.[17] Although this possibility cannot be ruled out, it seems less likely in view of the rapidity of the masoprocol effect. Obviously, this issue can be resolved experimentally. Finally, in light of evidence that another lipoxygenase inhibitor decreased blood pressure in rat models of hypertension,[5,6] it seems reasonable to propose at this time that the antihypertensive effects of phenidone and masoprocol are exerted through their function as lipoxygenase inhibitors. On the other hand, as masoprocol is also an antioxidant, this alternative mechanism of action cannot be excluded.

In conclusion, oral administration of masoprocol lowered blood pressure, as well as plasma insulin, free fatty acid, and triglyceride concentrations in fructose-fed rats. This experimental model of rodent hypertension replicates the metabolic abnormalities present in patients with essential hypertension that increase their risk of coronary heart disease.[18] As such, the potential use of masoprocol, or related lipoxygenase inhibitors, in the treatment of essential hypertension is self-evident.

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Exhibit 2

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Masoprocol decreases rat lipolytic activity by decreasing the phosphorylation of HSL

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Gowri, Maya S., Rakia K. Azhar, Fredric B. Kraemer, Gerald M. Reaven, and Salman Azhar. Masoprocol decreases rat lipolytic activity by decreasing the phosphorylation of HSL. *Am J Physiol Endocrinol Metab* 279: E593–E600, 2000.—Masoprocol (nordihydroguaiaretic acid), a lipoxygenase inhibitor isolated from the creosote bush, has been shown to decrease adipose tissue lipolytic activity both in vivo and in vitro. The present study was initiated to test the hypothesis that the decrease in lipolytic activity by masoprocol resulted from modulation of adipose tissue hormone-sensitive lipase (HSL) activity. The results indicate that oral administration of masoprocol to rats with fructose-induced hypertriglyceridemia significantly decreased their serum free fatty acid (FFA; $P < 0.05$), triglyceride (TG; $P < 0.001$), and insulin ($P < 0.05$) concentrations. In addition, isoproterenol-induced lipolytic rate and HSL activity were significantly lower ($P < 0.001$) in adipocytes isolated from masoprocol compared with vehicle-treated rats and was associated with a decrease in HSL protein. Incubation of masoprocol with adipocytes from chow-fed rats significantly inhibited isoproterenol-induced lipolytic activity and HSL activity, associated with a decrease in the ability of isoproterenol to phosphorylate HSL. Masoprocol had no apparent effect on adipose tissue phosphatidylinositol 3-kinase activity, but okadaic acid, a serine/threonine phosphatase inhibitor, blocked the antilipolytic effect of masoprocol. The results of these in vitro and in vivo experiments suggest that the antilipolytic activity of masoprocol is secondary to its ability to inhibit HSL phosphorylation, possibly by increasing phosphatase activity. As a consequence, masoprocol administration results in lower serum FFA and TG concentrations in hypertriglyceridemic rodents.

adipocyte; free fatty acid; triglyceride; hormone-sensitive lipase

HISTORICALLY, EXTRACTS OF the creosote bush (*Larrea tridentata*) have been extensively used by native healers throughout the Southwest region of North America for the treatment of type 2 diabetes. We have recently used an in vivo guided fractionation approach to identify masoprocol as the major, if not the only, compound in *L. tridentata* responsible for its antihyperglycemic effect. Masoprocol, also known as nordihydroguaiaretic acid, is a known lipoxygenase inhibitor (21, 34) and has been shown to lower plasma glucose concentrations and increase insulin-mediated glucose uptake in genetic models (*db/db* and *ob/ob*) of type 2 diabetes in mice (14).

In addition, rats were made insulin resistant and hyperinsulinemic by feeding a fat-enriched diet, and they were rendered hyperglycemic by injecting them with a modest amount of streptozotocin. In this situation, the insulin concentrations decline to levels similar to those in chow-fed rats and were no longer able to compensate for the insulin resistance, and hyperglycemia ensued (26). Masoprocol has been shown to effectively lower glucose concentrations in this nongenetic model of type 2 diabetes without any change in plasma insulin concentration but with decreases in free fatty acid (FFA) and triglyceride (TG) concentrations (26). In addition, masoprocol treatment has been shown to prevent hypertriglyceridemia in nondiabetic rats fed a fructose-enriched diet by significantly reducing hepatic TG secretion and increasing clearance of TG from the plasma (28).

Hormone-sensitive lipase (HSL) is a cytosolic neutral lipase that catalyzes the hydrolysis of intracellular TG (lipolysis) in adipose tissue (12, 32), skeletal muscle (20), and heart (31). HSL activity is regulated by multisite phosphorylation-dephosphorylation reactions in response to hormones (1, 29). Hormones (e.g., catecholamines) and other agonists that elevate cAMP levels stimulate HSL enzymatic activity through enhanced phosphorylation catalyzed by protein kinase A (2, 5). Insulin is thought to inhibit lipolysis by inactivating HSL due to the net dephosphorylation of the enzyme protein (5, 7). Okadaic acid, a polyether fatty acid, is a very potent inhibitor of protein phosphate 1 and protein phosphatase 2A (6), two of the four major protein phosphatases in cytosol of mammalian cells that catalyze hydrolysis of phosphoserine and phosphothreonine residues (4, 30). It is cell permeable and, when added to adipocytes, mimics the action of insulin in stimulating glucose transport (6, 9) and protein kinase activity (9).

In an effort to define the mechanism responsible for the metabolic effects of masoprocol, we have recently

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demonstrated that isoproterenol-induced lipolytic rate is significantly decreased when masoprocol is incubated with isolated adipocytes obtained from normal rats (8). The present studies were initiated to extend these observations and to specifically test the hypothesis that the ability of masoprocol to inhibit lipolytic activity is secondary to a direct effect on HSL.

MATERIALS AND METHODS

Animals and treatments. Male Sprague-Dawley rats obtained from Harlan Sprague-Dawley (Indianapolis, IN) were used in these studies. For in vitro studies, rats weighing ~220 g were fed Purina Rat Chow (no. 5012; St. Louis, MO) and water ad libitum and were maintained on a 12:12-h (0600–1800) light-dark cycle. On the day of the experiment, the animals were fasted for 4 h and decapitated by cervical dislocation, and epididymal fat pads were removed for preparation of adipocytes. For these studies, adipocytes were pooled from three to four animals. All experiments were performed in duplicate and were repeated at least three to six times (n = 3 or 6).

For in vivo studies, rats weighing 175–200 g were used. Rats were first maintained on a chow diet for ~1 wk and then were switched to a high-fructose diet (TD 78463; Harlan Teklad, Madison, WI) that provided 60% of total calories as fructose. After 10 days on the high-fructose diet, the degree of hypertriglyceridemia was evaluated by determining the total plasma TG levels. The hypertriglyceridemic animals (TG >250 mg/dl) were then divided into two groups (12 animals in each group, with comparable plasma TG concentrations). On *day 0* of treatment, the rats were fasted for 4 h, and tail vein blood was collected for baseline measurements of serum TG, glucose, insulin, and FFA. The two groups of rats were then treated with either vehicle (Gelucire 44/14) or masoprocol at a dose of 80 mg/k body wt two times a day for 4 days, delivered by oral gavage at a volume of 2.5 ml/kg body wt. During the treatment period, rats were maintained on high-fructose diets.

After 4 days of treatment, blood was collected from the tail vein, 3 h after the last dose of vehicle or masoprocol. Serum samples were used to measure TG and glucose concentrations by enzymatic calorimetric methods (15, 33) using Sigma Diagnostic kits (St. Louis, MO). Serum insulin concentrations were measured by RIA using a Linco Rat Insulin RIA kit (St. Charles, MO). FFA concentrations were measured using the nonesterified fatty acid (NEFA) C kit by the ACS-ACOD method following the instructions of the manufacturer. Rats were killed by decapitation, and epididymal fat pads were removed quickly and used for adipocyte isolation (to measure lipolytic activity) and quantitation of HSL activity. For Western blotting of HSL protein and RNA isolation, the tissue samples were collected and frozen immediately in liquid nitrogen. The tissues were stored at −80°C until used for various measurements.

Preparation of adipocytes. Adipocytes were prepared from the epididymal fat pads by a slight modification of the procedure of Rodbell (27) as previously described (24). In brief, fat pads were minced with scissors, placed in plastic flasks in Krebs bicarbonate buffer containing 3.5% BSA, 3 mM glucose, and 1 mg collagenase/ml, and incubated for 1 h at 37°C in a gyratory water-bath shaker. The released cells were washed three times in fresh Krebs buffer with 2% albumin and allowed to separate from the infranatant by flotation. Suitable aliquots of diluted cells were taken for measurement of rates of lipolytic activity. A 100-μl aliquot of diluted cells was also fixed in a solution of 2% osmium tetroxide in collidine buffer and was counted in a coulter counter (Hialeah, FL) for determination of cell number.

Measurement of rates of lipolytic activity. Rate of adipocyte lipolysis was determined using an established procedure of this laboratory (24). Aliquots of diluted cells (~1 × 10^5 cells/ml) were placed in plastic vials and preincubated in the presence or absence of 50 μM masoprocol in 1 ml of Krebs buffer containing 2% albumin at 37°C for 60 min, with continuous shaking at 40 counts/min. Subsequently, cells were incubated with or without (−)-isoproterenol (3 nM) or 8-(4-chlorophenylthio)-cAMP (8-CPT-cAMP, 0.5 mM) for 60 min at 37°C. In some instances, cells were also incubated with insulin (400 pM) for 60 min at 37°C with isoproterenol. At the end of the incubation, the cells were centrifuged, and the infranatant was collected for the quantification of glycerol and FFA, as described below. To further establish the specificity of masoprocol, the effect of esculetin, another specific inhibitor of lipoxygenase (18), was evaluated. Aliquots of adipocytes were preincubated with 4 or 40 μM esculetin for 60 min at 37°C and then were incubated with 3 nM isoproterenol for 60 min at 37°C to measure rates of lipolytic activity. For some other studies, adipocytes were also preincubated with 500 nM okadaic acid, a serine/threonine protein phosphatase inhibitor (6), or 1 μM wortmannin, a phosphatidylinositol 3-kinase (PI 3-kinase) inhibitor (19), for 15 min at 37°C before additional incubation with masoprocol and/or isoproterenol, as described above. Glycerol concentration in the infranatant was measured by an enzymatic method (23) using the TG kit. FFA concentration in the infranatant was measured using the NEFA C kit by the ACS-ACOD method.

To determine if masoprocol treatment could reduce lipolytic activity in studies performed ex vivo, the adipocytes were isolated from epididymal fat pads obtained from fructose-fed rats treated with vehicle or masoprocol. Adipocytes were incubated with isoproterenol (100 nM) for 60 min at 37°C. At the end of the incubation, the cells were centrifuged, and the infranatant was collected for the measurement of glycerol and FFA.

HSL activity. To assess the HSL protein and activity levels, adipose tissues from animals treated with masoprocol or vehicle or isolated adipocytes were homogenized using a Polytron in 50 mM Tris·HCl buffer (pH 7), 250 mM sucrose, and 5 μM EDTA. The homogenates were sequentially centrifuged at 1,500 (10 min) and 43,000 *g* (15 min) at 4°C (17). The clear supernatants (43,000 *g*) were used for measurement of HSL activity and HSL protein content by Western blotting. Protein content of supernatants was determined by a modification (22) of the technique of Lowry et al.

HSL activity was assayed as neutral cholesteryl esterase by following the release of [1-^{14}C]oleic acid from cholesteryl [1-^{14}C]oleate as described by Nakamura et al. (17) with minor modifications. The incubation medium in a final volume of 200 μl contained 100 nM potassium phosphate buffer, pH 7.4, 0.025% BSA, 1.25 nmol cholesteryl [1-^{14}C]oleate (~3 × 10^4 dpm) added in 4 μl acetone, and 10 μg supernatant. After incubation (10 min), the reaction was terminated (10) by addition of 1 ml of borate/carbonate buffer (0.1 M, pH 10.5) followed by 3 ml of chloroform-methanol-heptane (1.39: 1.28:1). The reaction tubes were vortexed vigorously for 1 min and centrifuged (1,500 *g* for 20 min at 10°C), and the released [1-^{14}C]oleate in the aqueous phase was determined by a scintillation counter. The results are expressed as picomoles [^{14}C]oleate released per minute per milligram protein.

Western blotting of HSL protein. An aliquot of adipose supernatant (25 μg protein) was mixed with equal volumes of 2× sample-loading buffer [4.6% (wt/vol) SDS, 16% (wt/vol) sucrose, 10% (vol/vol) β-mercaptoethanol, and 0.1 M

Table 1. *Effect of masoprocol treatment on serum glucose, insulin, triglyceride, and FFA concentrations in fructose-fed rats*

	Vehicle		Masoprocol	
	Baseline	Treatment	Baseline	Treatment
Body weight, g	259±2	258±4	256±3	250±13
Liver weight, g	N/A	9.99±0.3	N/A	9.79±0.4
Serum insulin, μU/ml	43±6	44±4	40±5	29±5*
Serum glucose, mg/dl	137±6	135±6	139±7	140±7
Serum triglycerides, mg/dl	224±25	219±32	242±15	79±5†‡
Serum FFA, meq/l	0.64±0.03	0.69±0.02	0.61±0.02	0.55±0.02*

Data are means ± SE. The two groups of rats (n = 12) were treated with either vehicle or masoprocol at a dose of 80 mg/kg twice a day for 4 days, delivered by oral gavage at a volume of 2.5 ml/kg body wt. FFA, free fatty acid. *P < 0.05 vs. vehicle. †P < 0.001 vs. vehicle. ‡P < 0.001 vs. masoprocol baseline. NA, not applicable.

Tris·HCl, pH 6.8], heated at 95°C for 5 min, and cooled to room temperature. The sample was subjected to electrophoresis on a 7% SDS-polyacrylamide gel (11). After electrophoretic separation, proteins were transferred to Immobilon-P membranes (Millipore, Bedford, MA) using standard techniques. The membranes were blocked in PBS-T buffer [pH 7.4; 10 mM sodium phosphate, 0.15 M NaCl, 2.5 mM $MgCl_2$, 0.1% Tween 20, and 5% (wt/vol) nonfat dried milk] for 1 h at 37°C. After being washed, the membranes were incubated with rabbit anti-HSL IgG (10) in blocking solution with agitation at 4°C overnight. Subsequently, the membranes were probed with horseradish peroxidase-conjugated goat anti-rabbit IgG. The signals corresponding to HSL were detected using the enhanced chemiluminescence system (Amersham, Arlington Heights, IL) according to the manufacturer's instructions. The Western blots were scanned by a densitometer.

Immunoprecipitation and detection of HSL phosphorylation. Anti-phosphoserine antibody was used to detect HSL phosphorylation. Aliquots of adipocyte extracts (200 μg protein) were first incubated with 5 μg of polyclonal anti-HSL in 2× immunoprecipitation buffer (2% Triton X-100, 300 mM NaCl, 20 mM Tris, pH 7.4, 2 mM EDTA, 2 mM EGTA, 4 mM sodium vanadate, 4.0 mM phenylmethylsulfonyl fluoride, and 1.0% Nonidet P-40) in a final volume of 1 ml for 2–3 h at 4°C. After incubation, protein A/G plus-agarose conjugate (50 μl) was added to each sample, and the tubes were vortexed and incubated with agitation overnight at 4°C. Immunoprecipitates were collected by centrifugation at 2,500 rpm for 5 min at 4°C. The pellets were carefully washed four times with 1× immunoprecipitation buffer as before. The pellets were resuspended in 25 μl of 2× electrophoresis buffer, boiled for 5 min, and centrifuged for 5 min at 2,500 rpm. The released supernatants were subjected to SDS-PAGE followed by Western blotting, as described above with some minor modifications. The primary antibody used for detecting HSL phosphorylation was rabbit anti-phosphoserine IgG at a dilution of 1:100.

RNA preparation. Approximately 1–2 g of adipose tissue, cut into small pieces, was homogenized in 5 ml of denaturing solution [4 M guanidium thiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% sarcosyl, and 0.1 M β-mercaptoethanol (added before use)] at room temperature using a Polytron (*setting 7* for 30 s). The solution was then extracted two times with an equal volume of chloroform to delipidate the sample (13). The aqueous phase was transferred to a polypropylene tube, and 0.1 ml of 2 M sodium acetate, pH 4, 1 ml of water-saturated phenol, and 0.2 ml of chloroform-isoamyl (49:1) mixture were sequentially added per 1 ml of aqueous phase. The RNA was then purified according to the procedure described by Chomczynski and Sacchi (3), dissolved in diethyl pyrocarbonate (DEPC)-treated water, and stored at −70°C.

Construction of HSL and 18S rRNA cDNA probes. A 470-bp *Apa* I-*Pst* I (position 687–1156) fragment from rat HSL cDNA was subcloned into the *Apa* I-*Pst* I sites of pBluescript KSII$^+$ (pBS KSII$^+$; Stratagene, La Jolla, CA). A 274-bp *Dra* II-*Tha* I (position 134–408) fragment of 18S ribosomal RNA cDNA (25) was subcloned into the *Dra* II-*Eco*R V sites of pBS KSII$^+$. The plasmids were linearized with appropriate restriction endonucleases (*Apa* I for HSL and *Bam*H I for 18S ribosomal RNA), and 3′-ends of the linearized HSL plasmids were filled using Klenow fragment, extracted two times with phenol and two times with chloroform, precipitated in ethanol, and redissolved in DEPC-treated water. The linearized plasmids were used for riboprobe synthesis.

Preparation of riboprobes. The antisense [^{32}P]cRNA probes were synthesized using [α-^{32}P]rCTP and the appropriate T3 or T7 RNA polymerase according to instructions supplied by

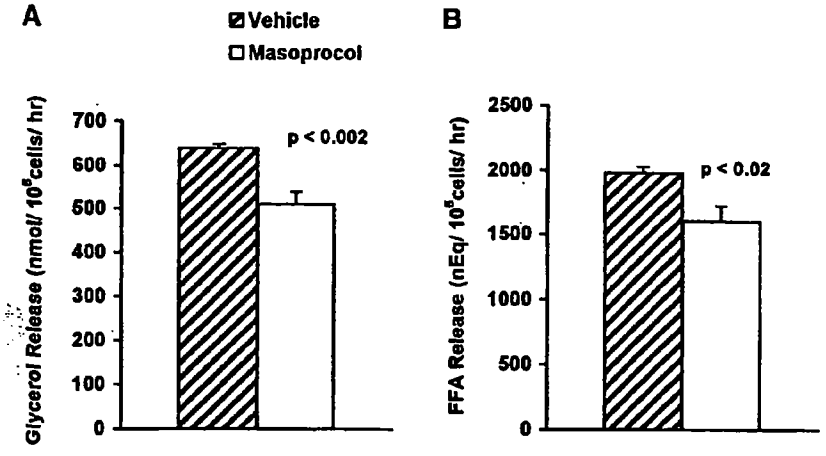

Fig. 1. Isoproterenol-induced glycerol (*A*) and free fatty acid (FFA; *B*) release by adipocytes isolated from fructose-fed rats treated with either vehicle or masoprocol. Isolated adipocytes were incubated with or without isoproterenol (100 nM) for 60 min at 37°C. After incubation, the media were collected and assayed for released glycerol and FFA. The basal rate of lipolysis in adipocytes from fructose-fed rats in the absence of added isoproterenol was 715 ± 8 nmol·10^{-5} cells·h^{-1}. In vivo administration of masoprocol did not alter basal lipolysis (787 ± 20 nmol·10^{-5} cells·h^{-1}). The results represent means ± SE of 9 individual measurements in each group.

the Strategene's in vitro transcription kit. Because of their high liability, the riboprobes were always freshly prepared before hybridization.

mRNA quantitation by RNase protection assay. The HSL mRNA levels were determined using a sensitive RNase protection assay. Aliquots of adipose tissue RNA or control tRNA (10 μg) were dried under vacuum and redissolved in 30 μl of hybridization buffer containing 10^4 cpm of probe [i.e., the radiolabeled HSL riboprobe or the 18S rRNA riboprobe that was used as an internal standard for quantification]. The mixture was incubated for 5 min at 85°C to denature RNA and was then rapidly transferred to a hybridization temperature of 42°C for incubation overnight (~16–18 h). The unprotected probe was hydrolyzed by digestion with 40 μg/ml RNase A and 2 μg/ml RNase T1 for 1 h at 30°C. The RNase digestion was terminated by the addition of proteinase K (50 μg) and SDS (2 mg) and incubation for 15 min at 37°C followed by phenol-chloroform extraction. The protected RNA-RNA hybrids were ethanol precipitated in the presence of yeast tRNA, and the pellets were dissolved in 15 μl of RNA loading buffer and heated for 5 min at 85°C. The protected fragments were separated on 6% acrylamide-urea denatur-

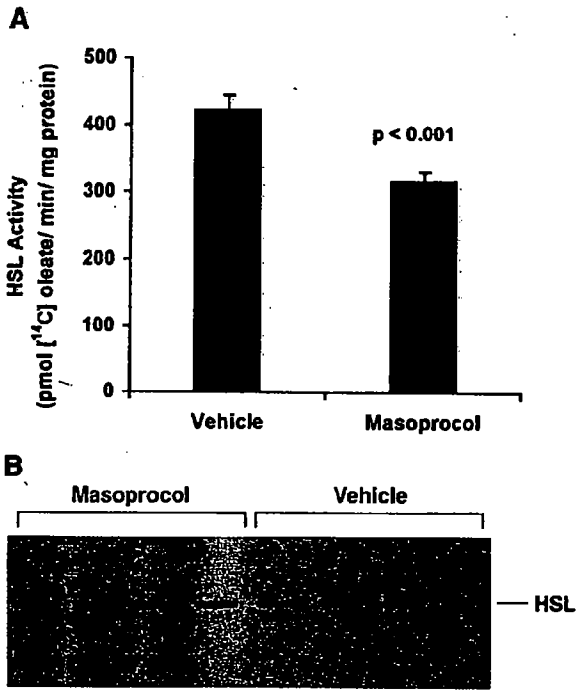

Fig. 2. Effects of masoprocol treatment on hormone-sensitive lipase (HSL) activity and protein content in adipose tissue extracts from fructose-fed rats. *A*: HSL activity. Groups of 12 fructose-fed animals were treated with either vehicle or masoprocol as described in *Animals and treatments*. The freshly prepared adipose tissue extracts were used for the measurement of HSL activity as described in MATERIALS AND METHODS. The results represent means ± SE of 12 individual rats in each group. *B*: Western blot analysis of HSL protein in adipose tissue from fructose-fed rats treated with either vehicle or masoprocol. The experimental details were the same as described in MATERIALS AND METHODS except 25 μg of protein were loaded in each lane. The four lanes under each vehicle and masoprocol treatment represent four individual animals.

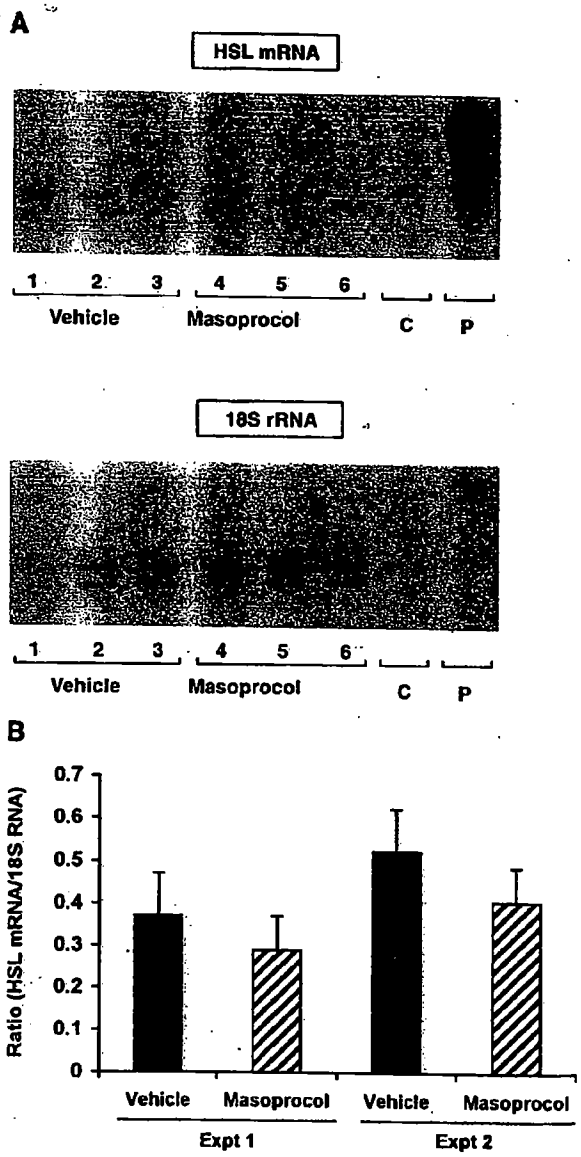

Fig. 3. *A*: analysis of adipose tissue HSL mRNA expression by RNase protection assay. Total RNA was isolated from adipose tissue of fructose-fed individual animals treated with either vehicle (*lanes 1–3*) or masoprocol (*lanes 4–6*). Aliquots of 10 μg RNA from various samples were subjected to RNase protection assay using ^{32}P-labeled HSL and 18S rRNA riboprobes. Other details were the same as described in MATERIALS AND METHODS. The protected fragments were resolved on 6% acrylamide-urea denaturing gels. After electrophoresis, gels were exposed to Kodak XAR-5 films at −70°C with intensifying screens. For quantitation, the signals from the films were analyzed by densitometry. C, tRNA control; P, probe. *B*: densitometric quantification of the bands shown in *A*. HSL mRNA is normalized to 18S rRNA as in *A*. Data represent experiments performed on 2 different occasions; each set includes 3 animals/vehicle and masoprocol-treated group.

ing gels. After electrophoresis, gels were exposed to Kodak XAR-5 film at −70°C with intensifying screens. For quantitation, the films were analyzed by densitometry. The data are expressed as a ratio of the HSL signal to that of 18S rRNA to correct for differences in loading the small amounts of RNA. In our studies, the levels of 18S rRNA remained constant in both vehicle- and masoprocol-treated rats.

Statistical analysis. Statistical significance of differences between the experimental groups was compared by ANOVA, and results are expressed as means ± SE.

Reagents. Masoprocol was obtained from either Sigma (St. Louis, MO) or Western Engineering and Research (El Paso, TX). Each batch of masoprocol was tested for glucose uptake in rat adipocytes to make sure that no difference existed between two sources or lots. Gelucire 44/14 was obtained from Gattefosse (Westwood, NJ). Type I collagenase was purchased from Worthington Biochemical (Freehold, NJ). BSA, (−)-isoproterenol, goat anti-rabbit IgG, TG kits (catalog nos. 339–10 and 320–10), and a Glucose Trinder Kit (catalog no. 315–100) were supplied by Sigma Chemical. The NEFA C kit (code no. 994–75409) was the product of Wako Chemicals (Richmond, VA). Esculetin, okadaic acid, and wortmannin were purchased from Biomol Research Laboratories (Plymouth Meeting, PA). Cholesteryl [1-^{14}C]oleate was purchased from American Radiolabeled Chemicals (St. Louis, MO). [α-^{32}P]CTP (specific activity 29.6 TBq/mmol; 800 Ci/mmol) was supplied by Du Pont (NEN Research Products, Boston, MA). Anti-phosphoserine antibody was obtained from Zymed (South San Francisco, CA). Protein A/G PLUS-Agarose was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

RESULTS

In vivo studies. Table 1 compares the effects of vehicle and masoprocol administration on fructose-induced changes in body weight, serum glucose, TG, FFA, and insulin concentrations. Body and liver weight were similar in the two groups of rats, as were the serum glucose concentrations. In contrast, serum FFA, TG, and insulin concentrations were significantly lower after masoprocol treatment but did not change in vehicle-treated rats.

Ex vivo studies. The prolipolytic effects of isoproterenol (100 nM) on adipocytes isolated from fructose-fed rats treated with vehicle or masoprocol are shown in Fig. 1. These results demonstrate that release of both glycerol ($P < 0.002$) and FFA ($P < 0.02$) by adipocytes from masoprocol-treated rat is decreased compared with adipocytes from rats receiving vehicle alone. HSL activity in adipocytes isolated from rats treated with vehicle or masoprocol is shown in Fig. 2*A*. The significant decrease ($P < 0.001$) in HSL activity in adipocytes from masoprocol-treated rats was associated with a concomitant fall in HSL protein, as determined by Western blot analysis (Fig. 2*B*).

Figure 3*A* shows the results obtained using a highly sensitive RNase protection assay to assess the levels of HSL mRNA in the adipose tissues of rats treated with vehicle or masoprocol. As a control, the expression of the stable marker 18S rRNA was also examined. The results presented in Fig. 3*B* (expressed as a ratio of HSL to 18S rRNA) demonstrate that expression of HSL mRNA was not significantly altered after exposure of rats to masoprocol and suggest the possibility that masoprocol modulates HSL activity posttranscriptionally.

In vitro studies. The next series of experiments were performed on adipocytes obtained from chow-fed rats, with masoprocol being added in vitro. The inhibitory effect of masoprocol and insulin on glycerol and FFA released during isoproterenol-induced lipolysis is shown in Fig. 4. It is clear from Fig. 4, *A* and *B*, that adipocytes pretreated with 50 μM masoprocol had a significant reduction in both glycerol (83 ± 3 vs. 165 ± 14 nmol·10^{-5} cells·h^{-1}; $P < 0.01$) and FFA (180 ± 30 vs. 440 ± 50 neq·10^{-5} cells·h^{-1}; $P < 0.02$) release compared with adipocytes incubated with isoproterenol alone. It can also be seen that this effect was comparable to the inhibition produced by 400 pM insulin. Masoprocol also significantly ($P < 0.05$) reduced 8 CPT-cAMP-induced lipolysis as shown in Fig. 5.

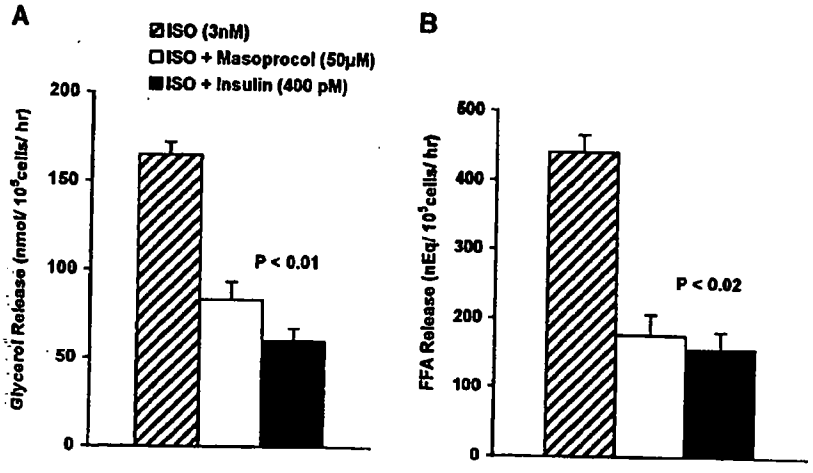

Fig. 4. Comparison of the ability of masoprocol and insulin to inhibit isoproterenol-induced lipolytic activity in adipocytes isolated from chow-fed rats. Isolated adipocytes were incubated with isoproterenol (ISO, 3 nM) with or without masoprocol (50 μM) or insulin (400 pM), and the amounts of glycerol and FFA released were quantified. Results represent means ± SE of 6 individual experiments done in duplicate on pooled adipocytes. The basal rate of lipolysis in adipocytes in the absence of isoproterenol was 66 ± 13 nmol·10^{-5} cells·h^{-1}. Pretreatment of adipocytes with masoprocol did not have any effect on the basal rate of lipolysis (65 ± 14 nmol·10^{-5} cells·h^{-1}).

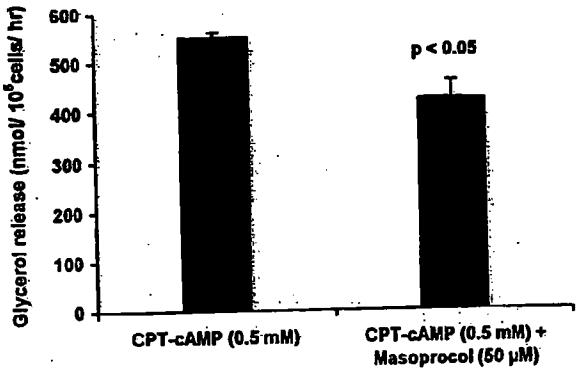

Fig. 5. Effect of masoprocol on 8-(4-chlorophenylthio)-cAMP (8 CPT-cAMP)-induced lipolytic activity in adipocytes isolated from chow-fed rats. The experimental details were as described in Fig. 4 except isoproterenol was replaced with 05 mM 8 CPT-cAMP. The results are means ± SE of 5 individual experiments performed in duplicate on pooled adipocytes.

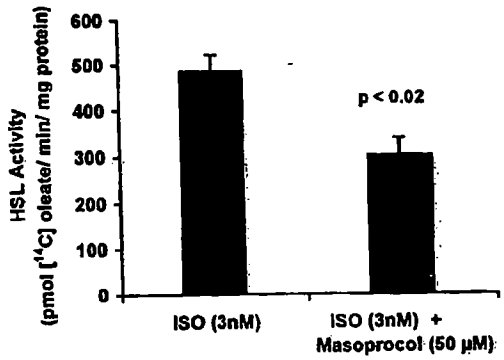

Fig. 7. Effect of in vitro addition of masoprocol on isoproterenol-induced stimulation of HSL activity in adipocytes isolated from chow-fed rats. Isolated adipocytes were preincubated with or without masoprocol for 60 min at 37°C, followed by a 60-min incubation with isoproterenol. The adipocyte samples were homogenized, and cellular extracts were employed for the measurement of HSL activity. Results represent means ± SE of 6 individual experiments done in duplicate on pooled adipocytes.

The effects of masoprocol and esculetin on isoproterenol-induced lipolytic activity in isolated adipocytes were also examined. As expected, pretreatment with 50 µM masoprocol significantly ($P < 0.01$) inhibited lipolytic activity, whereas esculetin (4 and 40 µM) did not (data not shown). The lipolytic effect of okadaic acid, a serine/threonine phosphatase inhibitor, in the presence or absence of masoprocol is shown in Fig. 6. Okadaic acid (500 nM) significantly enhanced isoproterenol-induced lipolytic activity ($P < 0.05$) by isolated adipocytes. Moreover, okadaic acid decreased the antilipolytic effect of masoprocol. Masoprocol (50 µM) inhibited isoproterenol-induced lipolytic activity by ~40%, but, in the presence of okadaic acid, masoprocol inhibited isoproterenol-induced lipolytic activity by only 5%. Wortmannin, a specific inhibitor of PI 3-kinase, did not affect the ability of masoprocol to inhibit isoproterenol-induced lipolytic activity (data not shown).

Figure 7 shows the in vitro inhibitory actions of masoprocol on isoproterenol-induced HSL activity in isolated adipocytes. In the presence of 3 nM isoproterenol, HSL activity was reduced by ~40% when adipocytes were preincubated with 50 µM masoprocol. Evidence that masoprocol decreases HSL activity by changing the phosphorylated state of HSL is presented in Fig. 8. In this experiment, an anti-phosphoserine antibody was used to detect the phosphorylation of HSL. The results clearly show that HSL protein immunoprecipitated from adipocytes treated with masoprocol had a decreased phosphoserine band.

DISCUSSION

This study was initiated to test the hypothesis that masoprocol inhibits the activity of HSL and that this

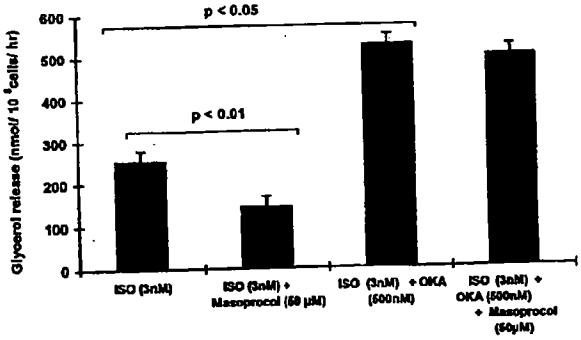

Fig. 6. Relationship between masoprocol and okadaic acid (OKA) modulation of isoproterenol-induced lipolytic activity in adipocytes isolated from chow-fed rats. The experimental conditions to measure lipolytic activity were as described in Fig. 4 and MATERIALS AND METHODS. The concentrations of isoproterenol, masoprocol, and okadaic acid used were 3 nM, 50 µM, and 500 nM, respectively. The results represent means ± SE of 3 individual experiments done in duplicate on pooled adipocytes.

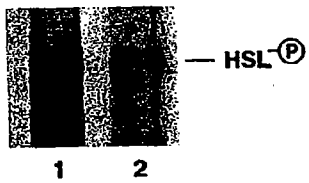

Fig. 8 In vitro effects of masoprocol on isoproterenol-stimulated phosphorylation of HSL protein. Adipocytes were sequentially treated with or without masoprocol followed by isoproterenol. Suitable aliquots of various cytosolic fractions (200 µg protein) were subjected to HSL immunoprecipitation with rabbit anti-HSL as described in MATERIALS AND METHODS. In each case, all of the immunoprecipitated HSL pellet was subjected to SDS-PAGE followed by Western blotting using the anti-phosphoserine and enhanced chemiluminescence detection system. The extent of HSL phosphorylation was determined by densitometric scanning of phospho (P)-HSL band. Under similar experimental conditions, the total amount of HSL protein (phosphorylated + nonphosphorylated HSL) was also determined using rabbit anti-rat HSL. *Lane 1*, adipocytes treated with isoproterenol alone; *lane 2*, adipocytes treated with masoprocol and isoproterenol.

action accounts for its ability to decrease the rate of adipocyte lipolysis and lower plasma FFA, TG, and insulin concentrations. The results presented not only provide strong support for this view, but they define a molecular mechanism to explain how masoprocol inhibits lipolytic activity.

At the simplest level, the studies of fructose-induced hypertriglyceridemia demonstrate that administration of masoprocol lowers plasma FFA and TG concentrations in nondiabetic rats, similar to earlier results demonstrating the same phenomenon in rats with an experimental form of type 2 diabetes (26). However, the two situations differ with regard to the effect of masoprocol administration on insulin and glucose concentrations. In both instances, there is evidence based on the serum insulin measurements that insulin sensitivity was enhanced after masoprocol administration. In the fructose-fed rat, the physiological response of the pancreatic β-cell to enhanced insulin sensitivity would be to secrete less insulin, leading to lower insulin but unchanged glucose concentrations, as shown in Table 1. In contrast, the masoprocol-induced increase in insulin sensitivity in the fat-fed/streptozotocin rat model of type 2 diabetes would lead to increased glucose disposal and a fall in glucose concentration. However, because these animals remain hyperglycemic, the pancreatic β-cell continues to secrete as much insulin as before. In addition to its effect on enhancing insulin sensitivity, the results presented indicated that isoproterenol-induced stimulation of adipocytes isolated from masoprocol-treated rats was associated with significantly less FFA and glycerol release. Finally, HSL activity was significantly lower in adipose tissue from masoprocol-treated rats, associated with a decrease in HSL protein.

The results of the in vivo administration of masoprocol raised the possibility that the antilipolytic effect of masoprocol was mediated via its ability to inhibit phosphorylation of HSL, and this conclusion received strong support from the in vitro effects of masoprocol on isolated adipocytes. More specifically, the results of the experiments with okadaic acid (a specific serine/threonine phosphatase inhibitor) and wortmannin (an inhibitor of PI 3-kinase) indicated that the antilipolytic effect of masoprocol was likely due to its stimulation of a serine/threonine phosphatase and not by stimulation of PI 3-kinase. The antilipolytic effect of masoprocol on isolated adipocytes was associated with a fall in HSL activity, and, by using as anti-phosphoserine antibody, we were able to show that this loss in activity was associated with a decrease in the phosphorylated state of HSL. This confirms that masoprocol may be stimulating a serine/threonine phosphatase via a second messenger pathway and may be causing dephosphorylation of HSL. Furthermore, the current in vivo and in vitro data suggest that masoprocol regulates HSL activity by a dual mechanism: a short-term effect on the phosphorylation state of HSL and a long-term effect on the HSL protein content.

Although a well-known lipoxygenase inhibitor, the profound metabolic effects of masoprocol only recently became apparent (8, 14, 26). The possibility that these effects may not be mediated by the lipoxygenase pathway must be considered, given the observation that esculetin, another lipoxygenase inhibitor, had no antilipolytic activity. In any event, the results of the present experiments confirm the ability of masoprocol to inhibit lipolysis in vitro (8), demonstrate that similar changes are seen after administration of masoprocol to rats with fructose-induced hypertriglyceridemia, and provide substantiative evidence that the metabolic effects of masoprocol are secondary to its ability to decrease HSL activity. Finally, these data, coupled with previous results (14, 26) showing that masoprocol can enhance insulin sensitivity and lower plasma FFA, glucose, and TG concentrations in hyperglycemic and/or hypertriglyceridemic rats, suggest that this may be a future therapeutic target of great interest.

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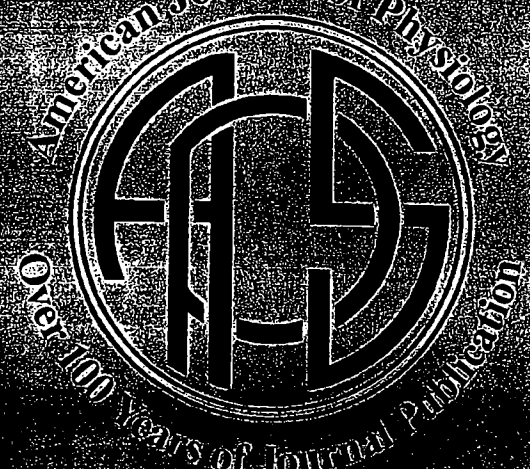

PART 1 OF TWO PARTS

PUBLISHED BY THE AMERICAN PHYSIOLOGICAL SOCIETY

Exhibit 3

United States Patent [19]

Copp et al.

[11] **Patent Number: 4,572,913**

[45] **Date of Patent: Feb. 25, 1986**

[54] **USE OF 3-(ARYLMETHYLENEAMINO)-1-ARYL-2-PYRAZOLINES IN THE PROPHYLAXIS AND TREATMENT OF INFLAMMATION, PAIN, PYRESIS, AND ASTHMA**

[75] Inventors: **Frederick C. Copp**, Beckenham; **Albert G. Caldwell**, West Wickham; **David Collard**, Beckenham, all of England

[73] Assignee: **Burroughs Wellcome Co.**, Research Triangle Park, N.C.

[21] Appl. No.: **331,009**

[22] Filed: **Dec. 15, 1981**

[30] **Foreign Application Priority Data**

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Mar. 18, 1981 [GB] United Kingdom 8108478

[51] **Int. Cl.**[4] **A61K 31/415**; A61K 31/44; A61K 31/47; C07D 231/06

[52] **U.S. Cl.** **514/403**; 514/313; 514/314; 514/333; 514/341; 548/379

[58] **Field of Search** ..... 542/414, 422, 424; 424/258, 263, 266, 273 P; 514/313, 314, 333, 341, 407; 548/379

[56] **References Cited**

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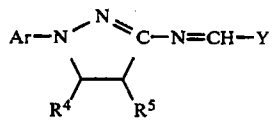
Duffin et al., J. Chem. Soc. 1954, pp. 408–415.
Kost et al., Fhur. Obshchei Khim. 29, pp. 498–502 (1959), [Translation and Abstract].

Primary Examiner—Richard A. Schwartz
Assistant Examiner—Kurt G. Briscoe
Attorney, Agent, or Firm—Donald Brown

[57] **ABSTRACT**

Compounds of formula (I)



(I)

inhibit both the cyclo-oxygenase and lipoxygenase pathways of arachidonic acid oxygenation and are useful in medicine as, e.g., anti-inflammatory and anti-asthmatic agents.

The compounds may be administered as the raw chemical or in association with a carrier as a pharmaceutical formulation.

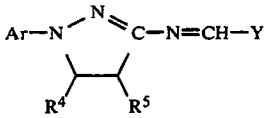
The compounds may be prepared by methods analogous to those known in the art, e.g., by the method of Duffin and Kendall in J. Chem. Soc. (1954), 408–415, or by other methods.

17 Claims, No Drawings

USE OF 3-(ARYLMETHYLENEAMINO)-1-ARYL-2-PYRAZOLINES IN THE PROPHYLAXIS AND TREATMENT OF INFLAMMATION, PAIN, PYRESIS, AND ASTHMA

This invention relates to heterocyclic compounds and their preparation and to the use of such compounds of pharmaceutical formulations thereof in medicine in a mammal, including man, as e.g. anti-inflammatory or anti-allergic agents or as agents in the prevention of tissue rejection.

Accordingly, the present invention relates to heterocyclic compounds of formula (I) and salts thereof:



(I)

wherein, Y is a monocyclic or bicyclic aromatic radical having from 5 to 10 ring atoms selected from carbon and nitrogen optionally substituted in any position of the ring by one or more substituent(s); R^4 and R^5 are each the same or different and are each selected from hydrogen, alkyl or Y as defined above; Ar is selected from Y as defined above with the proviso that Ar is other than unsubstituted phenyl.

Examples of Ar include substituted-phenyl, naphthyl, quinolyl and pyridyl. Particularly preferred aromatic radicals are substituted-phenyl and pyridyl, especially wherein 'pyridyl' is selected from 2-pyridyl and 4-pyridyl.

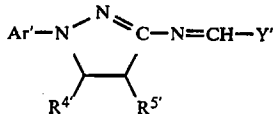
The aromatic ring is preferably substituted and examples of suitable substituents are halo, alkyl (which may itself be optionally substituted by halo), carboxy, alkoxy, nitro, amino (which may itself be optionally substituted by 1 or 2 alkyl groups), hydroxy and alkyl-sulphonyl of which the alkyl moiety may itself be optionally substituted by halo. Examples of especially suitable Ar substituents are halo (that is: fluoro, chloro, bromo and iodo) and trifluoromethyl. When Ar is substituted-phenyl, the preferred positions of the ring for the substituent(s) are those selected from the 2-, 3-, 4-, 3,4- and 2,6-positions. For example, Ar may be selected from 3-trifluoromethylphenyl, 4-trifluoromethylphenyl, 4-fluorophenyl, 4-chlorophenyl, 4-bromophenyl, 3-trifluoromethyl-4-fluorophenyl, 3-trifluoromethyl-4-chlorophenyl and 3-trifluoromethyl-4-bromophenyl. When Ar is pyridyl, the preferred position of the ring for any substituent is the 5-position. For example, Ar may be selected from 5-chloro-2-pyridyl,5-bromo-2-pyridyl and 5-iodo-2-pyridyl.

When any of R^4 and R^5 are Y the aromatic ring is preferably unsubstituted. For example, R^4 and R^5 may be selected from phenyl, 2-pyridyl and 4-pyridyl, but are preferably selected from hydrogen and alkyl.

Y is preferably a monocyclic aromatic radical having either from 3 to 7 ring atoms selected from carbon and nitrogen or a monocyclic or bicyclic aromatic radical of from 5 to 10 carbon atoms. Examples of such aromatic radicals are phenyl, pyridyl, naphthyl and pyrrolyl. When Y is substituted in the aromatic ring, the substituents may be selected from those examples described hereinbefore in the definition of 'Ar'. The preferred positions of the ring for any substituent are those selected from the 2-, 2,4- and 2,6-positions. For example,

Y may be selected from 2-hydroxyphenyl and 2,4-dihydroxyphenyl.

A subclass of the compounds of formula (I) are compounds of formula (IA)



(IA)

wherein

Y' is phenyl, pyridyl, pyrrolyl, naphthyl or quinolyl, each of which may optionally be substituted by one or more of halo, alkyl, alkoxy and hydroxy groups;

$R^{4'}$ and $R^{5'}$ are the same or different and are selected from hydrogen and alkyl; and

Ar' is pyridyl, quinolyl or substituted-phenyl, each of which pyridyl and quinolyl may be optionally substituted by one or more substituents, and the substituents are selected from halo, alkyl (which may itself be optionally substituted by halo), alkoxy and carboxyl groups.

Examples of compounds of formula (I) are:

3-salicylideneamino-1-(3-trifluoromethylphenyl)-2-pyrazoline;

3-benzylideneamino-1-(3-trifluoromethylphenyl)-2-pyrazoline;

3-(2,4-dihydroxybenzylideneamino)-1-(3-trifluoromethylphenyl)-2-pyrazoline;

3-(2-pyridylmethyleneamino)-1-(3-trifluoromethylphenyl)-2-pyrazoline;

3-(2-pyrrolylmethyleneamino)-1-(3-trifluoromethylphenyl)-2-pyrazoline;

3-salicylideneamino-1-(2-pyridyl)-2-pyrazoline;

3-(3-quinolylmethyleneamino)-1-(3-trifluoromethylphenyl)-2-pyrazoline;

3-(1-naphthylmethyleneamino)-1-(3-trifluoromethylphenyl)-2-pyrazoline;

3-(4-methylbenzylideneamino)-1-(2-naphthyl)-2-pyrazoline;

3-salicylideneamino-1-(3-quinolyl)-2-pyrazoline;

3-(4-chlorobenzylideneamino)-1-(4-chlorophenyl)-2-pyrazoline;

1-(4-bromo-3-trifluoromethylphenyl)-3-(2-hydroxybenzylideneamino)-2-pyrazoline;

1-(4-bromo-3-trifluoromethylphenyl)-3-(4-methoxybenzylideneamino)-2-pyrazoline;

3-benzylideneamino-1-(3-t-butylphenyl)-2-pyrazoline;

3-benzylideneamino-1-(3-trifluoromethylphenyl)-2-pyrazoline;

1-(5-bromo-6-methyl-2-pyridyl)-3-salicylideneamino-2-pyrazoline;

1-(5-bromo-6-methyl-2-pyridyl)-3-(1-naphthylmethyleneamino)-2-pyrazoline;

4-methyl-3-salicylideneamino-1-(3-trifluoromethylphenyl)-2-pyrazoline;

3-benzylideneamino-1-(4-methoxyphenyl)-2-pyrazoline;

3-benzylideneamino-1-(3-carboxylphenyl)-2-pyrazoline;

3-(2-hydroxy-1-naphthylmethyleneamino)-1-(3-trifluoromethylphenyl)-2-pyrazoline;

1-(2-chlorophenyl)-3-(2-hydroxy-1-naphthylmethyleneamino)-2-pyrazoline.

The compounds of formula (I) may be prepared by any method known in the art for the preparation of compounds of analogous structure, for example, by the

method of G. F. Duffin and J. D. Kendall in J. Chem. Soc. (1954), 408–415.

The compounds of formula (I) may be used in the relief of rheumatoid arthritis, rheumatoid spondylitis, osteroarthritis, gouty arthritis and other arthritic conditions; inflamed joints; eczema, other inflammatory skin conditions; inflammatory eye conditions including conjunctivitis; pyresis and other conditions associated with inflammation and pain. Such other conditions associated with inflammation include the reduction of tissue necrosis in chronic inflammation, the suppression of tissue rejection following transplant surgery and ulcerative colitis.

The compounds of formula (I) may also be used in the treatment or prophylaxis of allergic conditions and other airway inflammatory conditions such as asthma and of asthma having a non-allergic origin and bronchitis. The compounds may also be useful as antispasmogenic agents.

The amount required of a compound of formula (I) (herinafter referred to as the active ingredient) for therapeutic effect will, of course, vary both with the particular compound, the route of administration and the mammal under treatment. A suitable dose of a compound of formula (I) for a mammal suffering from an inflammatory, painful or pyretic condition as defined hereinbefore is 0.5 to 500 mg of base per kilogram bodyweight, the most preferred dosage being 0.5 to 50 mg/kg of mammal bodyweight, for example 5 to 25 mg/kg; administered two or three times daily.

In the case of the treatment or prophylaxis of inflammatory airway conditions, a suitable anti-asthmatic dose of a compound of formula (I) is 1 mg to 10 mg of base per kilogram bodyweight, the most preferred dosage being 1 mg to 5 mg/kg of mammal bodyweight, for example from 1 to 2 mg/kg.

While it is possible for an active ingredient to be administered alone as the raw chemical, it is preferable to present it as a pharmaceutical formulation. Conveniently, the active ingredient comprises from 0.1% to 99.9% by weight of the formulation. Conveniently, unit doses of a formulation contain between 0.1 mg and 1 g of the active ingredient. For topical administration, the active ingredient preferably comprises from 1% to 2% by weight of the formulation but the active ingredient may comprise as much as 10% w/w. Formulations suitable for nasal or buccal administration, (such self-propelling powder-dispensing formulations described hereinafter), may comprise 0.1 to 20% w/w, for example about 2% w/w of active ingredient.

The formulations, both for veterinary and for human medical use, of the present invention comprise an active ingredient in association with a pharmaceutically acceptable carrier therefor and optionally other therapeutic ingredient(s). The carrier(s) must be 'acceptable' in the sense of being compatible with the other ingredients of the formulations and not deleterious to the recipient thereof.

The formulations include those in a form suitable for oral, ophthalmic, rectal, parenteral (including subcutaneous, intramuscular and intravenous), intra-articular, topical, nasal or buccal administration.

The formulations may conveniently be presented in unit dosage form and may be prepared by any of the methods well known in the art of pharmacy. All methods include the step of bringing the active ingredient into association with the carrier which constitutes one or more accessory ingredients. In general, the formulations are prepared by uniformly and intimately bringing the active ingredient into association with a liquid carrier or a finely divided solid carrier or both, and then, if necessary, shaping the product into the desired formulation.

Formulations of the present invention suitable for oral administration may be in the form of discrete units such as capsules, cachets, tablets or lozenges, each containing a predetermined amount of the active ingredient; in the form of a powder or granules; in the form of a solution or a suspension in an aqueous liquid or non-aqueous liquid; or in the form of an oil-in-water emulsion or a water-in-oil emulsion. The active ingredient may also be in the form of a bolus, electuary or paste.

A tablet may be made by compressing or moulding the active ingredient optionally with one or more accessory ingredients. Compressed tablets may be prepared by compressing, in a suitable machine, the active ingredient in a free-flowing form such as a powder or granules, optionally mixed with a binder, lubricant, inert diluent, surface active or dispersing agent. Moulded tablets may be made by moulding, in a suitable machine, a mixture of the powdered active ingredient and a suitable carrier moistened with an inert diluent.

Formulations for rectal administration may be in the form of a suppository incorporating the active ingredient and a carrier such as cocoa butter, or in the form of an enema.

Formulations suitable for parenteral administration conveniently comprise a sterile aqueous preparation of the active ingredient which is preferably isotonic with the blood of the recipient.

Formulations suitable for intra-articular administration may be in the form of a sterile aqueous preparation of the active ingredient which may be in microcrystalline form, for example, in the form of an aqueous microcrystalline suspension. Liposomal formulations or biodegradable polymer systems may also be used to present the active ingredient for both intra-articular and ophthalmic administration.

Formulations suitable for topical administration include liquid or semi-liquid preparations such as liniments, lotions, applications; oil-in-water or water-in-oil emulsions such as creams, ointments or pastes; or solutions or suspensions such as drops. For example, for ophthalmic administration, the active ingredient may be presented in the form of aqueous eye drops as, for example, a 0.1–1.0% solution.

Formulations suitable for administration to the nose or buccal cavity include powder, self-propelling and spray formulations such as aerosols and atomizers. The formulations, when dispersed, preferably have a particle size in the range of 10 to 200u.

Such formulations are most preferably in the form of a finely comminuted powder for pulmonary administration from a powder inhalation device or self-propelling pwder-dispensing formulations, where the active ingredient, as a finely comminuted powder, may comprise up to 99.9% w/w of the formulation. In the case of self-propelling solution and spray formulations, the effect may be achieved either by choice of a valve having the desired spray characteristics (i.e. being capable of producing a spray having the desired particle size) or by incorporating the active ingredient as a suspended powder if controlled particle size. Thus the formulation, instead of passing into the lungs, is largely retained in the nasal cavity. These self-propelling formulations may be either powder-dispensing formulations or formula-

tions dispensing the active ingredient as droplets of a solution or suspension.

Self-propelling powder-dispensing formulations preferably comprise dispersed particles of solid active ingredient, and a liquid propellant having a boiling point if below 65° F. (18° C.) at atmospheric pressure. The liquid propellant may be any propellant known to be suitable for medicinal administration and may comprise one or more lower alkyl hydrocarbons or halogenated lower alkyl hydrocarbons or mixtures thereof; chlorinated and fluorinated lower alkyl hydrocarbons are especially preferred. Generally, the propellant constitutes 50 to 99.9% w/w of the formulation whilst the active ingredient constitutes 0.1 to 20% w/w, for example, about 2% w/w, of the formulation.

The pharmaceutically acceptable carrier in such self-propelling formulations may include other constituents in addition to the propellant, in particular a surfactant or a solid diluent or both. Surfactants are desirable since they prevent agglomeration of the particles of active ingredient and maintain the active ingredient in suspension. Especially valuable are liquid non-ionic surfactants and solid anionic surfactants or mixtures thereof. Suitable liquid non-ionic surfactants are those having a hydrophile-lipophile balance (HLB, see Journal of the Society of Cosmetic Chemists Vol. 1 pp. 311–326 (1949)) of below 10, in particular esters and partial esters of fatty acids with alphatic polyhydric alcohols, for instance, sorbitan monooleate and sorbitan trioleate, known commercially as 'Span 80' (Trade Name) and 'Span 85' (Trade Name), respectively. The liquid non-ionic surfactant may constitute from 0.01 up to 20% w/w of the formulation, though preferably it constitutes below 1% w/w of the formulation. Suitable solid anionic surfactants include alkali metal, ammonium and amine salts of dialkyl sulphosuccinate (where the alkyl groups have 4 to 12 carbon atoms) and alkyl benzene sulphonic acid (where the alkyl group has 8 to 14 carbon atoms). The solid anionic surfactants may constitute from 0.01 up to 20% w/w of the formulation, though preferably below 1% w/w of the composition solid diluents may be advantageously incorporated in such self-propelling formulations where the density of the active ingredient differs substantially from the density of the propellant; also, they help to maintain the active ingredient in suspension. The solid diluent is in the form of a fine powder, preferably having a particle size of the same order as that of the particles of the active ingredient. Suitable solid diluents include sodium chloride, sodium sulphate and sugars.

Formulations of the present invention may also be in the form of a self-propelling formulation wherein the active ingredient is present in solution. Such self-propelling formulations may comprise the active ingredient, propellant and co-solvent, and advantageously an antioxidant stabiliser. The propellant is one or more of these already cited above. Co-solvents are chosen for their solubility in the propellant, their ability to dissolve the active ingredient, and for their having the lowest boiling point consistent with these above-mentioned properties. Suitable co-solvents are lower alkyl alcohols and mixtures thereof. The co-solvent may constitute 5 to 40% w/w of the formulation, though preferably less than 20% w/w of the formulation. Antioxidant stabilisers may be incorporated in such solution-formulations to inhibit deterioration of the active ingredient and are conveniently alkali metal ascorbates or bisulphites. They are preferably present in an amount of up to 0.25% w/w of the formulation.

Such self-propelling formulations may be prepared by any method known in the art. For example, the active ingredient (either as particles as defined hereinbefore in suspension in a suitable liquid or in up to 20% w/w solution in an acceptable co-solvent, as appropriate) is mixed with any other constituents of a pharmaceutically acceptable carrier. The resulting mixture is cooled, introduced into a suitable cooled container and propellant is added thereto in liquid form; and the container is sealed. Alternatively, such self-propelling formulations may be prepared by mixing the active ingredient either in particles as hereinbefore defined or in 2 to 20% w/v alcohol or aqueous solution as appropriate, together with the remaining constituents of the pharmaceutically acceptable carrier other than the propellant; introducing the resulting mixture, optionally with some propellant, into a suitable container; and injecting the propellant, under pressure, into the container at ambient temperature through a valve which comprises a part of the container and is used to control release of the formulation from it. Desirably, the container is purged by removing air from it at a convenient stage in the preparation of the self-propelling formulation.

A suitable container for a self-propelling formulation is one provided with a manually-operable valve and constructed of aluminium, stainless steel or reinforced glass. The valve should, of course, be one having the desired spray characteristics of particle size as hereinbefore defined. Advantageously, the valve is of the type which delivers a fixed amount of the formulation on the occasion of each operation of the valve, for example, about 50 to 100 microliters of formulation in each delivery.

Formulations of the present invention may also be in the form of an aqueous or dilute alcoholic solution, optionally a sterile solution, of the active ingredient for use in a nebuliser or atomiser, wherein an accelerated air stream is used to produce a fine mist consisting of small droplets of the solution. Such formulations usually contain a flavouring agent such as saccharin sodium and a volatile oil. A buffering agent such as sodium metabisulphite and a surface active agent may also be included in such a formulation which should also contain a preservative such as methylhydroxybenzoate.

Other formulations suitable for nasal administration include a coarse powder having a particle size of 20 to 500 microns which is administered in the manner if which snuff is taken i.e. by rapid inhalation through the nasal pasage from a container of the powder held close up to the nose.

In addition to the aforementioned ingredients, the formulations of this invention may include one or more additional ingredients such as diluents, buffers, flavouring agents, binder, surface active agents, thickeners, lubricants, preservatives eg. methylhydroxybenzoate (including anti-oxidants), emulsifying agents and the like.

Any other therapeutic ingredient may comprise one or more of the following: anti-biotic, anti-fungal and anti-viral agents.

According to the present invention there are therefore provided:

(a) a novel compound of formula (I) or an acid addition salt thereof;

(b) a method for preparing a compound of formula (I);

(c) a pharmaceutical formulation comprising a non-toxic, effective arachidonic acid oxygenation inhibitory amount of a compound of formula (I) or a pharmaceutically acceptable salt thereof and a pharmaceutically acceptable carrier therefor;

(d) a method for preparing such formulations;

(e) a method for the prophylaxis or treatment of inflammation in a mammal, including man, comprising the administration to said mammal of a non-toxic, effective anti-inflammatory amount of a compound of formula (I);

(f) a method for the prophylaxis or treatment of pain in a mammal, including man, comprising the administration to said mammal of a non-toxic, effective analgesic amount of a compound of formula (I);

(g) a method for the prophylaxis or treatment of pyresis in a mammal, including man, comprising the administration to said mammal of a non-toxic, effective anti-pyretic amount of a compound for formula (I);

(h) a method for the prophylaxis or treatment of asthma in a mammal, including man, comprising the administration to said mammal of a non-toxic, effective, anti-asthmatic amount of a compound of formula (I);

(i) a method for the inhibition of a pathway of arachidonic acid oxygenation selected from the lipoxygenase and cyclo-oxygenase pathways, comprising the administration of a non-toxic, effective, inhibitory amount of a compound of formula (I) or a pharmaceutically acceptable acid addition salt thereof; and

(j) a compound of formula (I) for use in medicine in the inhibition of the lipoxygenase or cyclo-oxygenase pathways of arachidonic acid metabolism.

The following examples are provided by way of an illustration of the present invention and should in no way be construed as a limitation thereof. All temperatures indicated are in degrees Celsius.

EXAMPLE 1

Preparation of 3-(2,4'-carboxybutoxy-6-hydroxybenzylidine-amino)-1-(3-trifluoromethylphenyl)-2-pyrazoline

3-Amino-1-(3-trifluoromethylphenyl)-2-pyrazoline (438 mg) in methanol (4 ml) was mixed together with 2-4'-carboxybutoxy-6-hydroxybenzaldehyde (476 mg). A deep orange rapidly developed. The mixture was heated to reflux for 1½ hours during which time the reaction mixture set to a semi-solid mass. The mixture was cooled to 0° and the solid filtered off, washed with fresh methanol and dried in vacuo to produce 3-(2-4'-carboxybutoxy-6-hydroxybenzylideneamino)-1-(3-trifluoromethylphenyl)-2-pyrazoline, m.p. 179.1°.

Analysis: Required: C, 58.79; H, 4.93; N, 9.35; Found: C, 58.98; H, 5.00; N, 9.19.

EXAMPLE 2

Preparation of 3-(2-pyridylmethyleneamino)-1-(3-trifluoromethylphenyl)-2-pyrazoline

3-Amino-1-(3-trifluoromethylphenyl)-2-pyrazoline in methanol (23 ml) was stirred together with 2-pyridylaldehyde at room temperature for 2 hours and then under reflux for a further 2 hours. Thin layer chromatography (SiO_2, ethyl acetate) indicated that the reaction was substantially complete. After standing overnight, additional 2-pyridylaldehyde (0.5 g) was added and heating was continued for 2 hours. TLC again indicated that no further reaction had occurred. The reaction mixture was evaporated in vacuo to yield an orange-coloured solid which was stirred for 45 minutes in aqueous ethanol (3:1) and 3-(2-pyridylmethyleneamino)-1-(3-trifluoromethylphenyl)-2-pyrazoline was collected, m.p. 155.6°.

Analysis: $C_{16}H_{13}F_3N_4$; Required: C, 60.38; H, 4.11; N, 17.60; Found: C, 60.29; H, 4.18; N, 17.58.

EXAMPLE 3

Preparation of 3-(2-pyrrolylmethyleneamino)-1-(3-trifluoromethylphenyl)-2-pyrazoline

3-Amino-1-(3-trifluoromethylphenyl)-2-pyrazoline (0.6 g) in n-butanol (4 ml) was heated together with 2-pyrroylaldehyde (0.25 g) to 100° overnight under a nitrogen atmosphere. TLC (see example 1) indicated that a partial reaction had occurred. Heating was continued for a further 24 hours after which time little further reaction had occurred. The reaction mixture was left at room temperature under a nitrogen atmosphere for 4 days during which time dark-coloured crystals had formed which were collected and re-crystallized from aqueous isopropanol. The product was 3-(2-pyrrolylmethyleneamino)-1-(3-trilfuoromethylphenyl)-2-pyrazoline, m.p. 144°–145°.

Analysis: $C_{15}H_{13}F_3N_4$; Required: C, 58.82; H, 4.28; N, 18.29; Found: C, 59.04; H, 4.20; N, 18.5.

EXAMPLE 4

Preparation of 3-salicylidenamino-1-(3-trifluoromethylphenyl)-2-pyrazoline

A. A solution of 3-amino-1-(3-trifluoromethylphenyl)-2-pyrazoline (2.29 g) and salicylaldehyde (1.2 g) in methanol (22.9 ml) was heated to reflux for 30 minutes. The resulting semi-solid mass was cooled and then filtered to give an orange-coloured solid which was re-crystallized from ethanol to produce 3-salicylideneamino-1-(3-trifluoromethylphenyl)-2-pyrazoline m.p. 159.1° (yield 1.7 g).

Analysis: $C_{17}H_{14}F_3N_3O$; Required: C, 61.3; H, 4.1; N, 12.6; Found: C, 61.06; H, 4.36; N, 12.11.

B. Salicylaldehyde (80 mg) was added to a solution of 3-amino-1-(3-trifluoromethylphenyl)-2-pyrazoline hydrochloride (130 mg) in water (5 ml). The salicylaldehyde layer rapidly turned orange and after about 10 minutes a semi-solid mass had formed. Ethanol (1 ml) was added to give a clear solid. The mixture was kept overnight at room temperature and the product was collected and washed with water containing 5% ethanol and finally, dried in vacuo to produce 3-salicylideneamino-1-(3-trifluoromethylphenyl)-2-pyrazoline, m.p. 155.8° (yield 130 mg). The product was then re-crystallized with ethanol and on subsequent analysis, was found to be identical with that described in paragraph A.

EXAMPLE 5

Preparation of 3-salicylideneamino-1-(2-pyridyl)-2-pyrazoline

3-Amino-1-(2-pyridyl)-2-pyrazoline (1.6 g) and salicylaldehyde (1.2 g) were dissolved together with methanol (16 ml). The resulting solution was heated to reflux and after about 15 minutes a crystalline solid separated. After another 15 minutes at reflux the suspension was cooled and the separated 3-salicylideneamino-1-(2-pyridyl)-2-pyrazoline was re-

crystallized from methanol m.p. 242°–243° (yield 500 mg).

Analysis: $C_{15}H_{14}N_4O$; Required: C, 67.65; H, 5.3; N, 21.04; Found: C, 67.60; H, 5.34; N, 21.38.

EXAMPLE 6

Preparation of 3-(3-quinolylmethyleneamino)-1-(3-trifluoromethylphenyl)-2-pyrazoline

A mixture of 3-amino-1-(3-trifluoromethylphenyl)-2-pyrazoline (prepared in Reference Example 1 of our European patent specification No. 22-578) (0.73 g), 3-quinoline carboxaldehyde (0.5 g) and 1 drop glacial acetic acid in methanol (10 ml) was heated to reflux for thirty minutes. After cooling, the solid product was collected and recrystallized from ethanol, and subsequently from ethyl acetate and from toluene to afford 3-(3-quinolymethyleneamino)-1-(3-trifluoromethylphenyl)-2-pyrazoline, m.p. 209°–210°.

EXAMPLE 7

Preparation of 3-(1-naphthylmethyleneamino)-1-(3-trifluoromethylphenyl)-2-pyrazoline

A solution of 3-amino-1-(3-trifluoromethylphenyl)-2-pyrazoline (prepared in Reference Example 1 of our European patent specification No. 22-578) (10 g) and 1-naphthaldehyde (6.81 g) containing four drops of glacial acetic acid was heated to reflux in ethanol (50 ml) for twenty four hours. The resultant solid was collected and recrystallized from propan-1-ol to yield 3-(1-naphthylmethyleneamino)-1-(3-trifluoromethylphenyl)-2-pyrazoline, m.p.173°.

EXAMPLE 8

Preparation of 3-(4-methylbenzylideneamino)-1-(2-naphthyl)-2-pyrazoline

Example 8A: 3-amino-1-(2-naphthyl)-2-pyrazoline

2-Hydrazinonaphthalene (5 g) was added to a solution of sodium (0.7 g) in dried S.V.M. (20 ml) in a nitrogen atmosphere at 0°–5°. Acrylonitrile (1.8 g) was then added slowly and the resulting mixture allowed to attain room temperature over about 1 hour.

The mixture was then heated to reflux; after about 30 minutes it deposited a crystalline solid and after about 45 minutes a semi-solid mass had formed. After a total heating time of 1 hour, the mixture was allowed to cool and the solid filtered off with care.

The filtrate was deep purple but the residue was a clear yellow solid which was ground up with water, filtered and re-ground with S.V.M. After further filtering and grinding with S.V.M., the product was finally filtered, washed with S.V.M. and dried in vacuo to yield 4.4 g of 3-amino-1-(2-naphthyl)-2-pyrazoline, m.p. 190°–191°.

EXAMPLE 8B:

3-(4-methylbenzylideneamino)-1-(2-naphthyl)-2-pyrazoline

The amino compound prepared in Example 8A (500 mg) was suspended in S.V.M. (25 ml) together with 4-methylbenzaldehyde (300 mg) and 1 drop of glacial acetic acid. The mixture was stirred at reflux temperature for 2 hours. A dark orange solid formed and the reaction mixture was cooled and filtered to produce a dark yellow solid.

The product was washed with methanol and dried in vacuo to give 3-(4-methylbenzylideneamino)-1-(2-naphthyl)-2-pyrazoline, m.p. 184°–186°.

EXAMPLE 9

Preparation of 3-salicylideneamino-1-(3-quinolyl)-2-pyrazoline

3-Amino-1-(3-quinolyl)-2-pyrazoline (prepared in Example 10 of our co-pending application No. (A629)) (1.06 g, 0.005 mol) was added to a solution of salicylaldehyde (0.61 g, 0.005 mol) in methanol (8 ml). The mixture was refluxed for ½ hour and then the solid filtered off.

The solid was refluxed in methanol (200 ml) for ½ hour, the insoluble material filtered off and the product recrystallized from 2-ethoxyethanol with charcoaling to yield 0.14 g 3-salicylideneamino-1-(3-quinolyl)-2-pyrazoline, m.p. 288°–289°.

EXAMPLE 10

Preparation of 3-(4-chlorobenzylideneamino)-1-(4-chlorophenyl)-2-pyrazoline

3-Amino-1-(4-chlorophenyl)-2-pyrazoline (prepared in Reference Example 6 of our European patent specification No. 22-578) (1.95 g) in S.V.M. (5 ml) was mixed with excess 4-chlorobenzaldehyde (1.50 g) and the mixture was heated to reflux after the addition of 1 drop of glacial acetic acid. A virtually clear solution formed which rapidly crystallised to form a bright orange product which was collected, washed with S.V.M. and dried in vacuo to produce 2.95 g 3-(4-chlorobenzylideneamino)-1-(4-chlorophenyl)-2-pyrazoline, m.p. 193°–195° (decomp).

EXAMPLE 11

Preparation of 1-(4-bromo-3-trifluoromethylphenyl)-3-(2-hydroxybenzylideneamino)-2-pyrazoline

3-Amino-1-(4-bromo-3-trifluoromethylphenyl)-2-pyrazoline (prepared in Example 39 of our European patent specification No. 22-578) (140 mg) and salicylaldehyde (100 mg) were dissolved together in methanol (2 ml) and 1 drop of glacial acetic acid. The mixture was heated to reflux for 1 hour. During this time a deep orange-red colour developed and the mixture crystallised. It was kept at 0° for 3 hours, then the solid was collected, washed with methanol and dried in vacuo to yield 150 mg 1-(4-bromo-3-trifluoromethylphenyl)-3-(2-hydroxybenzylideneamino)-2-pyrazoline, m.p. 170° (decomp).

EXAMPLE 12

Preparation of 1-(4-bromo-3-trifluoromethylphenyl)-3-(4-methoxybenzylideneamino)-2-pyrazoline

3-Amino-1-(4-bromo-3-trifluoromethylphenyl)-2-pyrazoline (prepared in Example 39 of our European patent specification No. 22-578) (240 mg) in S.V.M. (5 ml) together with 4-methoxybenzaldehyde (160 mg) and 1 drop of glacial acetic acid were heated to reflux for 1 hour. A yellow product separated which was collected, washed with methanol and dried in vacuo to yield 300 mg 1-(4-bromo-3-trifluoromethylphenyl)-3-(4-

methoxybenzylideneamino)-2-pyrazoline, m.p. 175°–176° (decomp).

EXAMPLE 13

3-Amino-1-(3-t-butylphenyl)-2-pyrazoline

3-t-Butylaniline hydrochloride (5 g) in concentrated hydrochloric acid (8 ml) was stirred at 0° whilst a solution of sodium nitrite (1.86 g) in water (2.4 ml) was slowly added. The mixture was kept at 0° for 1 hour and, after filtering (at 0°), it was treated dropwise with a solution of stannous chloride dihydrate (18.2 g) in concentrated hydrochloric acid (18.8 ml). A pink-coloured solid separated. After 1 hour, this solid was filtered off and washed with saturated aqueous sodium chloride. The resulting salt was converted into base in the usual way to give 3-t-butylphenylhydrazine, b.p. 88°–90°/0.25 mm Hg.

This hydrazine (1.2 g) was then added to a solution of sodium (0.029 g) in ethanol (1.5 ml) at room temperature in an atmosphere of nitrogen. The resulting solution was cooled to −10°, acrylonitrile (0.24 ml) added and the mixture heated to reflux for 5 hours. The solid which separated on cooling was recrystallized from light petroleum (b.p. 80°–100°) to give 3-amino-1-(t-butylphenyl)-2-pyrazoline, m.p. 113.5° (yield 648 mg).

EXAMPLE 14

3-benzylideneamino-1-(3-t-butylphenyl)-2-pyrazoline

3-Amino-1-(3-t-butylphenyl)-2-pyrazoline (500 mg) was reacted with benzaldehyde (500 mg) in boiling methanol (5 ml) in the presence of glacial acetic acid (1 drop). After some 4 hours, the mixture was cooled and 3-benzylideneamino-1-(3-t-butylphenyl)-2-pyrazoline separated in crystals m.p. 140°–141°, (yield 500 mg).

EXAMPLES 15 to 22

By a method analogous to that described in detail in the foregoing Examples were also prepared the following:

EXAMPLE 15

3-Benzylideneamino-1-(3-trifluoromethylphenyl)-2-pyrazoline, m.p. 162°–163°.

EXAMPLE 16

1-(5-Bromo-6-methyl-2-pyridyl)-3-salicylideneamino-2-pyrazoline, m.p. 215°–216°.

EXAMPLE 17

1-(5-Bromo-6-methyl-2-pyridyl)-3-(1-naphthylmethyleneamino)-2-pyrazoline, m.p. 199°.

EXAMPLE 18

4-Methyl-3-salicylideneamino-1-(3-trifluoromethylphenyl)-2-pyrazoline, m.p. 105°–106°.

EXAMPLE 19

3-Benzylideneamino-1-(4-methoxyphenyl)-2-pyrazoline, m.p. 199°–200°.

EXAMPLE 20

3-Benzylideneamino-1-(3-carboxylphenyl)-2-pyrazoline

3-Amino-1-(3-carboxyphenyl)-2-pyrazoline (850 mg) in methanol (10 ml) was treated with benzaldehyde (870 mg) and glacial acetic acid (1 drop). The mixture was heated to reflux for 7 hours, then left to cool overnight. The resultant product was filtered off and recrystallised from n-propanol to yield 3-benzylideneamino-1-(3-carboxyphenyl)-2-pyrazoline, m.p. 185°.

EXAMPLE 21

3-(2-Hydroxy-1-naphthylmethyleneamino)-1-(3-trifluoromethylphenyl)-2-pyrazoline, m.p. 247°–249°.

EXAMPLE 22

1-(2-Chlorophenyl)-3-(2-hydroxy-1-naphthylmethyleneamino)-2-pyrazoline, m.p. 195°–197°.

EXAMPLE A

Tablet

In one tablet	
Active Ingredient	5.0 mg
Lactose	82.0 mg
Starch	10.0 mg
Povidone	2.0 mg
Magnesium stearate	1.0 mg

Mix together the active ingredient, lactose and starch. Granulate the powders using a solution of povidone in purified water. Dry the granules, add the magnesium stearate and compress to produce tablets, 100 mg per tablet.

EXAMPLE B

Ointment

Active Ingredient	1.0 g
White soft paraffin to	100.0 g

Disperse the active ingredient in a small volume of the vehicle. Gradually incorporate this into the bulk to produce a smooth, homogeneous product. Fill into collapsible metal tubes.

EXAMPLE C

Cream for Topical Use

Active Ingredient	1.0 g
Polawax GP 200	20.0 g
Lanolin Anhydrous	2.0 g
White Beeswax	2.5 g
Methyl Hydroxybenzoate	0.1 g
Distilled Water to	100.0 g

Heat the polawax, beeswax and lanolin together at 60°. Add a solution of methyl hydroxybenzoate. Homogenise using high speed stirring. Allow the temperature to fall to 50°. Add and disperse the active ingredient. Allow to cool with slow speed stirring.

EXAMPLE D

Lotion for Topical Use

Active Ingredient	1.0 g
Sorbitan Monolaurate	0.6 g
Polysorbate 20	0.6 g
Cetostearyl Alcohol	1.2 g
Glycerin	6.0 g
Methyl Hydroxybenzoate	0.2 g
Purified Water to	100.0 ml

The methyl hydroxybenzoate and glycerin were dissolved in 70 ml of the water at 75° C. The sorbitan monolaurate, polysorbate 20 and cetostearyl alcohol were melted together at 75° and added to the aqueous solution. The resulting emulsion was homogenised, allowed to cool with continuous stirring and the active ingredient added as a suspension in the remaining water. The whole was stirred until homogenous.

EXAMPLE E

Eye Drops

Active Ingredient	0.5 g
Methyl Hydroxybenzoate	0.01 g
Propyl Hydroxybenzoate	0.04 g
Purified Water B.P. to	100.00 ml

The methyl and propyl hydroxybenzoates were dissolved in 70 ml purified water at 75° and the resulting solution then allowed to cool. The active ingredient was added next and the solution made up to 100 ml with purified water. The solution was sterilised by filtration through a membrane filter 0.22 um pore size and packed aseptically into suitable sterile containers.

EXAMPLE F

Injection Solution

Active Ingredient	10.0 mg
Water for Injections B.P.	1.0 ml

The active ingredient was dissolved in half of the Water for Injections and then made up to volume and sterilised by filtration. The resulting solution was distributed into ampoules under asceptic conditions.

EXAMPLE G

Inhibition of Lipoxygenase and Cyclo-oxygenase

In an enzyme assay according to the method of G. Blackwell and R. J. Flower (Br.J.Pharmac., 63: 36O(1978)), compounds of the invention were found to have an IC_{50} (uM) for inhibition of each of lipoxygenase and cyclo-oxygenase as indicated in Table I:

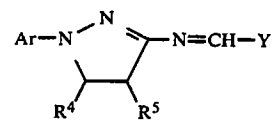
TABLE I

Compound	IC_{50} (μM) Cyclo-oxygenase	Lipoxygenase
of Example 1	<3	10–20
of Example 2	1	~3
of Example 3	<1	1
of Example 4	~3	~3
of Example 7	10	6
of Example 8	5	12
of Example 11	~5	>10
of Example 12	~1	~1
of Example 13	<1	<1
of Example 15	~3	<1
of Example 18	~1	~1

What we claim is:

1. 3-Salicylideneamino-1-(3-trifluoromethylphenyl)-2-pyrazoline.

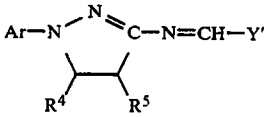
2. A pharmaceutical formulation useful in treating inflammation in mammals comprising an effective amount of a compound of formula (I) or a pharmaceutically acceptable salt thereof:



(I)

wherein, Y is a monocyclic or bicyclic aromatic radical selected from phenyl, naphthyl, quinolyl, and pyridyl optionally substituted in any position of the ring by one or more substituent(s) selected from fluoro, chloro, bromo or iodo, nitro, carboxy, hydroxy, amino, monoalkyl substituted-amino, dialkyl substituted-amino, monohalo substituted-alkyl, dihalo substituted-alkyl, trihalo substituted-alkyl, alkyl, alkoxy, carboxyalkoxy, alkylsulphonyl, monohalo substituted-alkyl sulphonyl, dihalo substituted-alkyl sulphonyl and trihalo substituted-alkyl sulphonyl; R^4 and R^5 are each the same or different and are each selected from hydrogen, alkyl, or Y as defined above; and Ar is selected from Y as defined above with the proviso that Ar is other than unsubstituted phenyl.

3. A formulation according to claim 2 in which the compound or a pharmaceutically acceptable salt thereof is of formula (IA):



(IA)

wherein, Y is phenyl, pyridyl, naphthyl or quinolyl, each of which may optionally be substituted by one or more of halo, alkyl, alkoxy and hydroxy groups; R^4 and R^5 are the same or different and are each selected from hydrogen and alkyl; and Ar is pyridyl, quinolyl or substituted-phenyl, each of which pyridyl and quinolyl may be optionally substituted by one or more substituents, and the substituents are selected from halo, alkyl (which may itself be optionally substituted by halo), alkoxy and carboxyl groups.

4. A pharmaceutical formulation useful in treating inflammation in humans comprising an effective amount of a compound selected from:

3-salicylideneamino-1-(3-trifluoromethylphenyl)-2-pyrazoline;

3-benzylideneamino-1-(3-trifluoromethylphenyl)-2-pyrazoline;

3-(2,4-dihydroxybenzylideneamino)-1-(3-trifluoromethylphenyl)-2-pyrazoline;

3-(2-pyridylmethyleneamino)-1-(3-trifluoromethylphenyl)-2-pyrazoline;

3-salicylideneamino-1-(2-pyridyl)-2-pyrazoline;

3-(3-quinolylmethyleneamino)-1-(3-trifluoromethylphenyl)-2-pyrazoline;

3-(1-naphthylmethyleneamino)-1-(3-trifluoromethylphenyl)2-pyrazoline;

3-(4-methylbenzylideneamino)-1-(2-naphthyl)-2-pyrazoline;

3-salicylideneamino-1-(3-quinolyl)-2-pyrazoline;

3-(4-chlorobenzylideneamino)-1-(4-chlorophenyl)-2-pyrazoline;

3-(2-hydroxybenzylideneamino)-1-(4-bromo-3-trifluoromethylphenyl)-2-pyrazoline;

3-(4-methoxybenzylideneamino)-1-(4-bromo-3-trifluoromethylphenyl)-2-pyrazoline;

3-benzylideneamino-1-(3-t-butylphenyl)-2-pyrazoline;

3-salicylideneamino-1-(5-bromo-6-methyl-2-pyridyl)-2-pyrazoline;
3-(1-naphthylmethyleneamino)-1-(5-bromo-6-methyl-2-pyridyl)-2-pyrazoline;
4-methyl-3-salicylideneamino-1-(3-trifluoromethylphenyl)-2-pyrazoline;
3-benzylideneamino-1-(4-methoxyphenyl)-2-pyrazoline;
3-benzylideneamino-1-(3-carboxyphenyl)-2-pyrazoline;
3-(2-hydroxy-1-naphthylmethyleneamino)-1-(3-trifluoromethylphenyl)-2-pyrazoline;
3-(2-hydroxy-1-naphthylmethyleneamino)-1-(2-chlorophenyl)-2-pyrazoline or a pharmaceutically acceptable salt thereof.

5. A formulation according to claim **2** in unit dosage form.

6. A formulation according to claim **2** in the form of capsules, tablets, suppositories, liniments, lotions, creams ointments, drops or aerosols.

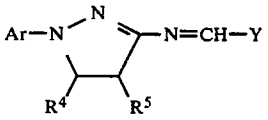
7. A formulation according to claim **2** in a form suitable for ophthalmic administration.

8. A formulation according to claim **2** in the form of aqueous eye drops.

9. A formulation according to claim **2**, wherein the compound or salt of formula (I) is further in association with another therapeutic ingredient selected from antibiotic, anti-fungal and anti-viral agents.

10. A pharmaceutical formulation useful in treating inflammation in mammals comprising an effective amount of 3-salicylideneamino-1-(3-trifluoromethylphenyl)-2-pyrazoline or a pharmaceutically acceptable salt thereof.

11. A method for prophylaxis or treatment of inflammation in a mammal in need thereof, including man, comprising the administration to said mammal of a non-toxic, effective anti-inflammatory amount of a compound of formula (I) or a pharmaceutically acceptable salt thereof:



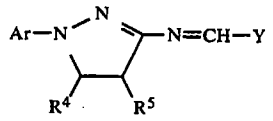
(I)

wherein, Y is a monocyclic or bicyclic aromatic radical selected from phenyl, naphthyl, quinolyl, and pyridyl optionally substituted in any position of the ring by one or more substituent(s) selected from fluoro, chloro, bromo or iodo, nitro, carboxy, hydroxy, amino, monoalkyl substituted-amino, dialkyl substituted-amino, monohalo substituted-alkyl, dihalo substituted-alkyl, trihalo substituted-alkyl, alkyl, alkoxy, carboxyalkoxy, alkylsulphonyl, monohalo substituted-alkyl sulphonyl, dihalo substituted-alkyl sulphonyl and trihalo substituted-alkyl sulphonyl; R^4 and R^5 are each the same or different and are each selected from hydrogen, alkyl, or Y as defined above; and Ar is selected from Y as defined above with the proviso that Ar is other than unsubstituted phenyl.

12. A method for the prevention or treatment of inflammation in a mammal in need thereof comprising the administration to said mammal of 3-salicylideneamino-1-(3-trifluoromethylphenyl)-2-pyrazoline or a pharmaceutically acceptable salt thereof.

13. The method of claim **12** in which the mammal is a human.

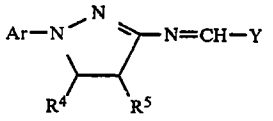
14. A method for the prophylaxis or treatment of pain in a mammal in need thereof, including man, comprising the administration to said mammal of a non-toxic, effective analgesic amount of a compound of formula (1) or a pharmaceutically acceptable salt thereof:



(I)

wherein, Y is a monocyclic or bicyclic aromatic radical selected from phenyl, naphthyl, quinolyl, and pyridyl optionally substituted in any position of the ring by one or more substituent(s) selected from fluoro, chloro, bromo or iodo, nitro, carboxy, hydroxy, amino, monoalkyl substituted-amino, dialkyl substituted-amino, monohalo substituted-alkyl, dihalo substituted-alkyl, trihalo substituted-alkyl, alkyl, alkoxy, carboxyalkoxy, alkylsulphonyl, monohalo substituted-alkyl sulphonyl, dihalo substituted-alkyl sulphonyl and trihalo substituted-alkyl sulphonyl; R_4 and R^5 are each the same or different and are each selected from hydrogen, alkyl, or Y as defined above; and Ar is selected from Y as defined above with the proviso that Ar is other than unsubstituted phenyl.

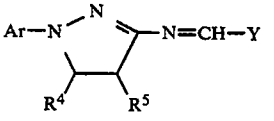
15. A method of inhibiting the lipoxygenase or cyclooxygenase pathways of arachidonic acid metabolism in a mammal in need thereof comprising the administration of an effective inhibitory amount of a compound of formula (I) or a pharmaceutically acceptable salt thereof:



(I)

wherein, Y is a monocyclic or bicyclic aromatic radical selected from phenyl, naphthyl, quinolyl, and pyridyl optionally substituted in any position of the ring by one or more substituent(s) selected from fluoro, chloro, bromo or iodo, nitro, carboxy, hydroxy, amino, monoalkyl substituted-amino, dialkyl substituted-amino, monohalo substituted-alkyl, dihalo substituted-alkyl, trihalo substituted-alkyl, alkyl, alkoxy, carboxyalkoxy, alkylsulphonyl, monohalo substituted-alkyl sulphonyl, dihalo-substituted-alkyl sulphonyl and trihalo substituted-alkyl sulphonyl; R_4 and R_5 are each the same or different and are each selected from hydrogen, alkyl, or Y as defined above; and Ar is selected from Y as defined above with the proviso that Ar is other than unsubstituted phenyl.

16. A method for the prophylaxis or treatment of pyresis in a mammal in need thereof, including man comprising the administration to said mammal of an effective amount of a compound of formula (I) or a pharmaceutically acceptable salt thereof:

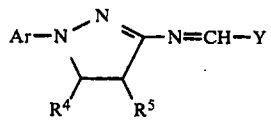


(I)

wherein, Y is a monocyclic or bicyclic aromatic radical selected from phenyl, naphthyl, quinolyl, and pyridyl optionally substituted in any position of the ring by one

or more substituent(s) selected from fluoro, chloro, bromo or iodo, nitro, carboxy, hydroxy, amino, monoalkyl substituted-amino, dialkyl substituted-amino, monohalo substituted-alkyl, dihalo substituted-alkyl, trihalo substituted-alkyl, alkyl, alkoxy, carboxyalkoxy, alkylsulphonyl, monohalo substituted-alkyl sulphonyl, dihalo substituted-alkyl sulphonyl and trihalo substituted-alkyl sulphonyl; R^4 and R^5 are each the same or different and are selected from hydrogen, alkyl, or Y as defined above; and Ar is selected from Y as defined above with the proviso that Ar is other than unsubstituted phenyl.

17. A method for the prophylaxis or treatment of asthma in a mammal in need thereof comprising the administration to a mammal of an antiasthmatic amount of a compound of formula (I) or a pharmaceutically acceptable salt thereof:



(I)

wherein, Y is a monocyclic or bicyclic aromatic radical selected from phenyl, naphthyl, quinolyl, and pyridyl optionally substituted in any position of the ring by one or more substituent(s) selected from fluoro, chloro, bromo or iodo, nitro, carboxy, hydroxy, amino, monoalkyl substituted-amino, dialkyl substituted-amino, monohalo substituted-alkyl, dihalo substituted-alkyl, trihalo substituted-alkyl, alkyl, alkoxy, carboxyalkoxy, alkylsulphonyl, monohalo substituted-alkyl sulphonyl, dihalo substituted-alkyl sulphonyl and trihalo substituted-alkyl sulphonyl; R^4 and R^5 are each of the same or different and are each selected from hydrogen, alkyl, or Y as defined above; and Ar is selected from Y as defined above with the proviso that Ar is other than unsubstituted phenyl.

* * * * *

Exhibit 4

Masoprocol Decreases Serum Triglyceride Concentrations in Rats With Fructose-Induced Hypertriglyceridemia

Karen A. Scribner, Theresa M. Gadbois, Maya Gowri, Salman Azhar, and Gerald M. Reaven

Historically, extracts of the creosote bush have been used by native healers of the Southwest region of North America to treat symptoms of type 2 diabetes. More recently, we have shown that masoprocol (nordihydroguaiaretic acid), a pure compound isolated from the creosote bush (*Larrea tridentata*), decreases serum glucose and triglyceride (TG) levels when administered orally in rodent models of type 2 diabetes. The present studies were undertaken to determine if masoprocol also decreases TG concentrations in rats with fructose-induced hypertriglyceridemia (HTG), a nondiabetic model of HTG associated with insulin resistance and hyperinsulinemia. Serum TG levels, which were significantly higher after rats ate a fructose-enriched (60% by weight) diet for 14 days as compared with chow-fed controls (411 *v* 155 mg/dL, $P < .01$), decreased in a stepwise fashion in fructose-fed rats treated orally with masoprocol for 4 to 8 days over a dose range of 10 to 80 mg/kg twice daily. Using the nonionic detergent Triton WR 1339 to compare TG secretion rates in masoprocol- and vehicle-treated rats, masoprocol at a dose of 40 or 80 mg/kg twice daily, significantly reduced hepatic TG secretion ($P < .01$) and liver TG content ($P < .001$), whereas lower doses of masoprocol decreased serum TG without an apparent reduction in hepatic TG secretion. Administration of Intralipid (a fat emulsion) showed that the half-time for removal of TG from serum was also shorter in masoprocol-treated rats versus vehicle-treated controls (31 *v* 64 minutes, $P < .05$). In addition adipose tissue lipoprotein lipase (LPL) activity was increased in masoprocol-treated rats and adipose tissue hormone-sensitive lipase (HSL) activity was decreased. We conclude that masoprocol administration to rats with fructose-induced HTG results in lower serum TG levels associated with reduced hepatic TG secretion and increased peripheral TG clearance.

MASOPROCOL (nordihydroguaiaretic acid) is a pure compound isolated from the creosote bush (*Larrea tridentata*), which has been recently shown to decrease serum glucose and triglyceride (TG) concentrations in rodent models of type 2 diabetes.[1,2] The rodent models used in these studies are insulin-resistant and hyperinsulinemic, features characteristic[3-5] of rats with fructose-induced hypertriglyceridemia (HTG). Based on these similarities, we initiated the present study to determine if masoprocol also decreases plasma TG concentrations in a nondiabetic rat model with dietary-induced HTG.

MATERIALS AND METHODS

Six-week-old male Sprague-Dawley rats from Charles River Laboratories (Hollister, CA) were used for all experiments. The rats weighed 125 to 175 g upon arrival and were housed 3 to 4 per cage in a light- and temperature-controlled animal facility with ad libitum tap water and either standard chow or a diet containing 60% (by weight) fructose (TD 78463) from Harlan Teklad (Madison, WI). After approximately 2 weeks on the fructose-enriched diet, rats were screened for HTG (TG > 250 mg/dL) by obtaining blood from the tail vein 2 to 5 hours after food was removed. Based on the results, the animals were divided into groups with comparable plasma TG concentrations and used to assess the effects of masoprocol administration in the subsequent experimental protocol.

Following the creation of 2 or more experimental groups with similar plasma TG concentrations, the rats were treated with vehicle or masoprocol at a dose of 10 to 80 mg/kg twice daily for 8 days by oral gavage at a vol of 2.5 mL/kg body weight. Masoprocol was obtained from Sigma (St Louis, MO) or from Western Engineering and Research (El Paso, TX) and was formulated in Gelucire 44/14 (Gattefosse, Westwood, NJ). Gelucire 44/14 is a waxy solid at room temperature, and it therefore required heating to a liquid state before the addition of masoprocol. The mixture was vortexed and then sonicated in a bath sonicator at 50° to 55°C until the masoprocol dissolved completely. The control solution and the one containing masoprocol were maintained in a warm water bath while gavaging the animals, to keep the solution in a liquid state.

After several days (4 to 8) of treatment with masoprocol or vehicle, blood was collected by tail snip once per day 3 hours after the first dose of vehicle or masoprocol, and the serum was assayed for TG, glucose, insulin, and free fatty acid (FFA). Blood samples were collected in Microtainer (Becton Dickinson, Franklin Lakes, NJ) serum separator tubes and centrifuged at 12,000 rpm for 10 minutes. The serum was removed and TG and glucose concentrations were measured immediately by enzymatic colorimetric methods[6,7] using Sigma diagnostic kits. Insulin concentrations were measured in previously frozen and thawed serum samples by radioimmunoassay using Linco Rat Insulin Radioimmunoassay kits (St. Charles, MO). FFA concentrations were measured by an enzymatic colorimetric method using Wako NEFA C test kits (Richmond, VA).

Triton WR 1339, a nonionic detergent that prevents TG removal, was obtained from Sigma to measure the hepatic secretion rate of TG[8,9] 3 hours after the final dose of vehicle or masoprocol. Triton WR 1339 (600 to 800 mg/kg) was diluted with saline to a concentration of 300 mg/mL, vortexed, and sonicated with heat (60° to 70°C) until the solution was homogeneous. Blood samples were collected from conscious animals before and at 60 and 120 minutes after injection of Triton WR 1339 (2.0 mL/kg) into a catheterized tail vein. The accumulation of TG in the serum following injection with Triton WR 1339 was used as a measure of the hepatic secretion rate (milligrams per deciliter per hour).

The TG removal rate was estimated, using Intralipid (Vitrum, Stockholm, Sweden) to quantify the half-time ($t_{1/2}$) for TG removal,[10,11] 3 hours after the final dose of vehicle or masoprocol. Blood samples were collected from conscious animals before and at 1, 10, 30, 60, and 90 minutes after injection of Intralipid (2.0 mL/kg or 400 mg/kg lipid) into a catheterized tail vein. The WinNonlin pharmacokinetic modeling program (Scientific Consulting, Cary, NC) was used to determine the $t_{1/2}$ of TG removal using a single-compartment, 1st-order model of elimination.

Lipoprotein lipase (LPL) activity was measured in tissue extracts of

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doi:10.1053/meta.2000.8604

soleus and epididymal fat tissue collected from rats treated with vehicle or masoprocol (80 mg/kg twice daily) for 5 days. The animals were anesthetized with pentobarbital 3 hours after the morning dose of vehicle or masoprocol. Soleus and adipose tissue samples were collected, rapidly frozen in liquid nitrogen, and stored at −80°C until assay for LPL activity. The assay method described previously by Nilsson-Ehle and Schotz[12] was used. Briefly, a stable emulsion of ^{14}C-triolein (in toluene) was obtained by sonication in glycerol to produce the concentrated substrate. Extracts of soleus (60 to 80 mg) and epididymal fat (100 to 150 mg) tissue were obtained by homogenizing tissue samples in a solution of Tris hydrochloride containing 1% bovine serum albumin (BSA), heparin (10 U/mL), 0.5% deoxycholate, and 0.02% Nonidet P-40. The tissue homogenates were centrifuged (12,000 rpm for 30 minutes) and the supernatant was removed and incubated for 1 hour in concentrated substrate diluted 1:10 with Tris hydrochloride, dH_2O, and 15% BSA. Incubations were performed in the presence and absence of serum from fasted rats, and specific LPL activity was determined as the difference in activity between these 2 conditions.

The TG content of the liver was measured in animals treated with vehicle or masoprocol (80 mg/kg twice daily) for 4 days. Measurement methods for the lipids were described by Brown et al.[13] Briefly, tissues were homogenized in homogenization buffer (0.3 mol/L sucrose, 25 mmol/L 2-mercaptoethanol, and 10 mmol/L EDTA, pH 7.0). An aliquot was transferred to stoppered tubes for extraction of the lipids. Methanol containing 30,000 dpm [^{3}H]cholesterol was added (to monitor recovery) and the tubes were vortexed vigorously. Chloroform was added, mixed, and allowed to stand for 1 hour at room temperature. Then, equal volumes of chloroform and 0.15 mol/L NaCl were added to separate the phases, and the tubes were allowed to stand overnight at 4°C. The tubes were centrifuged lightly to ensure that separation of the phases was complete.[14] The lower organic phase was removed as completely as possible by aspiration, and the aqueous phase was washed once with chloroform. The combined lower phase was dried under nitrogen. The lipids were resuspended in 95% ethanol and 100 µL was removed for liquid scintillation counting of [^{3}H]cholesterol (to correct for recovery). TG content in the lipid extract was measured by the method of Pinter et al[15] using a kit from Sigma Diagnostics and is expressed as micromoles of TG per gram of tissue.

Hormone-sensitive lipase (HSL) activity was measured in adipose tissue collected from rats treated with vehicle or masoprocol (80 mg/kg twice daily) for 4 days. The adipose tissue was homogenized using a Polytron (Brinkman Instruments, Westbury, NY) on setting 3 in 50 mmol/L Tris hydrochloride buffer (pH 7) containing 250 mmol/L sucrose and 5 µmol/L EDTA. The homogenate was centrifuged at 1,500× g for 10 minutes at 4°C, followed by ultracentrifugation at 43,000× g for 15 minutes at 4°C.[16] The protein concentration was determined on the clear supernatant (43,000× g) by the modified Lowry technique,[17] and aliquots were used for measurement of HSL activity. HSL activity was measured as neutral cholesteryl esterase by determining the release of [1-^{14}C]oleic acid from cholesteryl [1-^{14}C]oleate as described previously by Nakamura et al,[16] with minor modifications. Briefly, the supernatant of adipocytes (10 µg) and the substrate (approximately 3×10^4 dpm/1.22 nmol cholesteryl oleate/4 µL acetone) were incubated at 37°C for 10 minutes in 100 mmol/L potassium phosphate buffer (pH 7.0) containing 0.025% BSA (total vol, 200 µL).[16] The reaction was terminated by the addition of 1 mL borate/carbonate buffer (0.1 mol/L, pH 10.5) followed by 3 mL chloroform:methanol: heptane (1.39:1.28:1).[18] The reaction tubes were vortexed vigorously for 1 minute and centrifuged (1,500× g for 20 minutes at 10°C). The amount of [1-^{14}C]oleate released in the aqueous phase was determined by scintillation counting using a Beckman (Fullerton, CA) LS3800 scintillation counter and is expressed as picomoles of [1-^{14}C]oleate released per minute per milligram of protein.

Statistical Analysis

Results are presented as the mean ± SEM. Statistical comparisons were made using Student's t test for unpaired samples or a 1-way ANOVA as appropriate, with post hoc comparisons using Fisher's protected least-significant difference test, except for LPL activity, which was analyzed using the Mann-Whitney test for nonparametric unpaired samples.

RESULTS

The effects of the fructose-enriched diet for 2 weeks on postabsorptive serum concentrations of glucose, insulin, TG, and FFA are shown in Table 1. The results show that body weight and serum glucose were lower in fructose-fed versus chow-fed rats. In contrast, serum insulin ($P < .02$), TG ($P < .001$), and FFAs ($P < .001$) were higher in fructose-fed rats.

Serum TG concentrations in fructose-fed rats treated with vehicle or masoprocol (10 to 80 mg/kg twice daily) for 4 to 8 days are shown in Fig 1. Masoprocol was administered twice per day at a dose of 10, 20, and 40 mg/kg (for 8 days) and 80 mg/kg (for 4 days). These results show that masoprocol produced a significant decrease ($P < .01$) in serum TGs compared with vehicle treatment and the decrease was dose-dependent: the greater the dose of masoprocol, the greater the decrease in TG concentration. The masoprocol-induced decrease in TGs was also associated with a significant decline in the FFA concentration (2.4 ± 0.2 v 1.4 ± 0.08 mEq/L, $P < .01$) but a comparable insulin concentration (49 ± 3 v 49 ± 4 µU/mL).

The effect of masoprocol treatment on the rate of hepatic TG secretion as estimated by intravenous administration of Triton WR 1339 is shown in Fig 2. These studies were performed in the same animals as shown in Fig 1, 3 to 4 hours after the final dose of vehicle or masoprocol. Treatment with masoprocol 40 or 80 mg/kg twice daily led to a significant decrease ($P < .01$) in the rate of hepatic TG secretion as compared with vehicle. However, lower doses of masoprocol (10 and 20 mg/kg twice daily) did not affect TG secretion. To determine if the masoprocol-induced decrease in hepatic TG secretion was associated with accumulation of fat in the liver, the TG content was determined in liver tissue obtained from rats (n = 12) treated with either masoprocol (80 mg/kg twice daily) or vehicle. These measurements showed that, if anything, hepatic TG content was lower in masoprocol-treated rats (7.6 ± 0.3 v 9.6 ± 0.7 µmol/g, $P < .05$).

The effect of masoprocol treatment on the $t_{1/2}$ for TG removal is shown in Fig 3. Animals were treated with vehicle or

Table 1. Effect of a Fructose-Enriched Diet on Serum Glucose, Insulin, TG, and FFA Concentrations

Variable	Fructose-Fed (n = 12)	Chow-Fed (n = 12)	P
Weight (g)	253 ± 5	304 ± 6	<.001
Glucose (mg/dL)	124 ± 2	131 ± 2	<.05
Insulin (µU/mL)	49 ± 4	36 ± 2	<.02
TG (mg/dL)	411 ± 53	155 ± 10	<.001
FFA (mEq/L)	1.6 ± 0.09	0.8 ± 0.04	<.001

NOTE. Data are the mean ± SEM. Animals were fed a fructose-enriched diet or regular chow for 20 days.

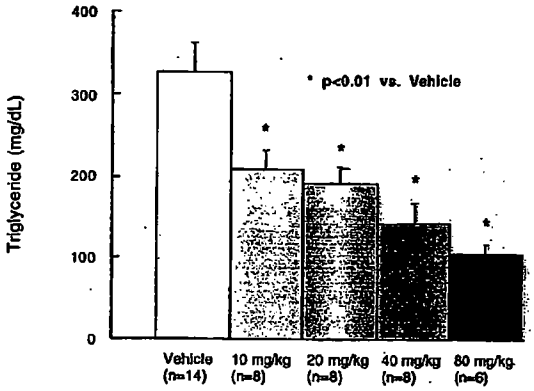


Fig 1. Postabsorptive serum TG concentration 8 days after the twice-daily oral administration of vehicle or varying amounts of masoprocol. All 4 doses of masoprocol significantly decreased plasma TG ($P < .01$). The number of animals in each group is given in parentheses.

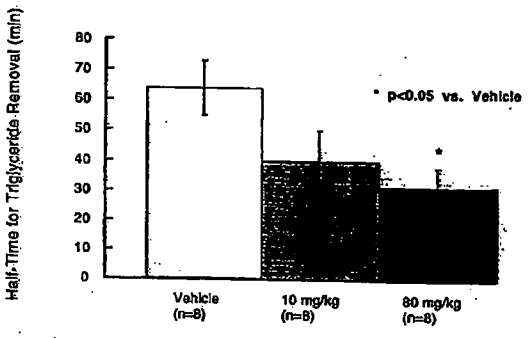
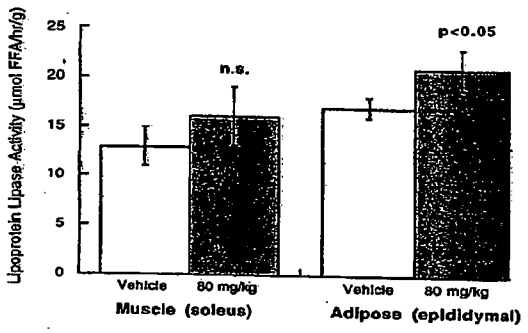


Fig 3. Estimates of the rate of TG removal from the plasma in fructose-fed rats treated for 8 days with a twice-daily dose of vehicle or masoprocol (10 and 80 mg/kg). The half-time for TG removal was determined by measuring the plasma TG concentration at frequent intervals following injection of a lipid emulsion (Intralipid). There were 6 to 8 rats in each group, and the half-time was significantly lower ($P < .05$) for fructose-fed rats with the higher dose of masoprocol.

masoprocol (10 or 80 mg/kg twice daily) for 8 days prior to determination of peripheral TG removal following the injection of Intralipid. The mean $t_{1/2}$ for TG removal was reduced by masoprocol treatment (10 mg/kg, 40 minutes; 80 mg/kg, 31 minutes) compared with vehicle treatment (64 minutes), although it was significant ($P < .05$) only at the higher dose.

Figure 4 compares the activity of LPL in soleus and epididymal fat tissue and adipose tissue HSL in tissue extracts from animals treated with vehicle or masoprocol (80 mg/kg twice daily) for 8 days. LPL activity was increased in both muscle and adipose tissue, but the difference was significant only in adipose tissue ($P < .05$). HSL activity in adipose tissue was lower ($P < .001$) in masoprocol-treated animals.



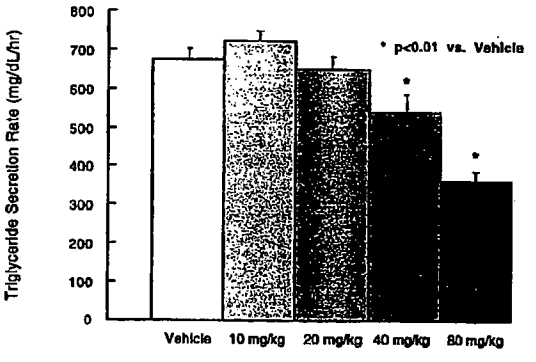


Fig 2. Estimates of TG secretion rates in fructose-fed or vehicle-treated rats after 4 days (80 mg/kg) or 8 days (vehicle and 10, 20, and 40 mg/kg) of twice-daily administration of test substance. TG secretion was estimated by determining the difference in plasma TG secretion before and 2 hours after administration of Triton WR 1339. There were 6 to 8 rats in each group, and the TG secretion rate was significantly lower v vehicle ($P < .01$) in rats treated with either 40 or 80 mg/kg masoprocol.

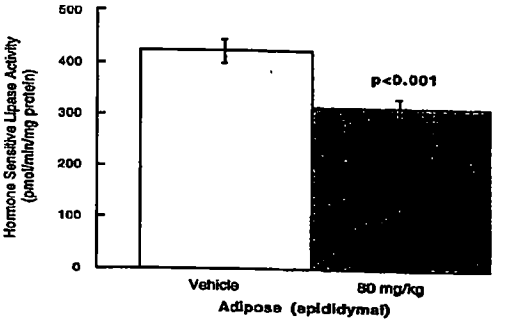


Fig 4. LPL activity in soleus muscle and epididymal adipose tissue and HSL activity in adipose tissue of fructose-fed rats 8 days after twice-daily oral administration of either vehicle or 80 mg/kg masoprocol. There were 6 to 8 rats in each group.

DISCUSSION

The results of the current study have again shown that HTG develops when rats consume a fructose-enriched diet. As in previous studies,[3-5] the fructose-induced increase in the plasma TG concentration was associated with hyperinsulinemia without a substantial change in the serum glucose concentration. In addition, the results demonstrate that masoprocol dramatically decreases serum TG concentrations in rats with fructose-induced HTG, extending prior observations that TGs decreased when rats with an experimental form of type 2 diabetes were treated with masoprocol.[2] Thus, the TG-lowering effect of masoprocol can be discerned irrespective of the initial serum glucose concentration.

In addition to showing that masoprocol decreases the serum TG concentration in rats with fructose-induced HTG, the data provide insight as to how this is accomplished. More specifically, evidence has been presented that masoprocol both inhibits hepatic TG secretion and increases the TG removal rate. If attention is first directed to the conclusion that masoprocol decreases hepatic TG secretion, the experimental data are most consistent with the view that this is secondary to the antilipolytic effect of masoprocol. In the first place, the fact that hepatic TG secretion was lower in masoprocol-treated rats suggests that the associated decrease in serum TGs was due to decreased TG secretion. Furthermore, based on the results of hepatic perfusion studies,[19] the decrease in FFA concentrations in masoprocol-treated rats in the presence of similar insulin concentrations would result in a decrease of hepatic TG secretion. Finally, the decrease in HSL activity in fructose-fed rats treated with masoprocol provides an explanation at the cellular level for the lower FFA concentrations. Thus, it is possible to propose the following coherent formulation to account for the dramatic ability of masoprocol to reduce serum TG concentrations in fructose-fed rats: masoprocol decreases adipose tissue HSL activity, resulting in lower circulating FFA concentrations, leaving less FFA available for hepatic TG synthesis, leading to a decrease in hepatic TG secretion and lower serum TG concentrations.

Although the evidence is indirect, it appears that the ability of masoprocol to reduce serum TG concentrations is not entirely due to a decrease in hepatic TG secretion. For example, plasma TGs decreased significantly in fructose-fed rats treated with masoprocol 10 and 20 mg/kg twice daily, doses that were not associated with a significant decrease in hepatic TG secretion. By inference, it could be argued that masoprocol also has the ability to enhance the rate of removal of TG-rich lipoproteins from the plasma. Providing some support for this possibility is the observation that the removal rate of plasma TG was increased following injection of Intralipid in masoprocol-treated rats. In addition, the increases in LPL activity were consistent with an effect of masoprocol on TG catabolism. Although only the increase in adipose tissue LPL was statistically significant, the increase in soleus muscle activity was of the same order of magnitude, and the combined effect on both tissues might well be viewed as offering support for the view that masoprocol enhances the rate of TG removal from plasma.

In conclusion, administration of masoprocol, a well-known lipoxygenase inhibitor, can profoundly decrease serum TG concentrations in fructose-fed rats in a dose-dependent manner. At least some of this effect appeared to be due to a decrease in hepatic TG secretion, secondary to an increase in adipose tissue HSL and a decrease in serum FFAs. However, masoprocol may also enhance TG catabolism, and this effect may contribute to its ability to reduce serum TG concentrations. On the other hand, evidence in support of this alternative is not as strong, and other possibilities have not been excluded, ie, a decrease in intestinal fat absorption. A definitive answer as to the mechanism by which masoprocol decreases serum TGs in fructose-fed rats will depend on the results of further experiements. Finally, masoprocol is a well-known lipoxygenase inhibitor, and it is not clear whether its effect on TG metabolism is related to this action. In that context, there is evidence that various amine-carboxyboranes can both decrease plasma TGs in rodents and inhibit lipoxygenase activity.[20] Although it is possible that lipoxygenase inhibitors might offer a useful approach for the treatment of HTG in humans, it may be that this effect is masoprocol-specific and unrelated to the inhibition of lipoxygenase pathways. Irrespective of the answer to this question, the extreme potency of masoprocol to reduce serum TG concentrations makes it worthy of further study.

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Exhibit 5

US006191169B1

(12) **United States Patent**
Nadler et al.

(10) **Patent No.: US 6,191,169 B1**
(45) **Date of Patent: *Feb. 20, 2001**

(54) **HUMAN LEUKOCYTE 12-LIPOXYGENASE AND ITS ROLE IN THE PATHOGENESIS OF DISEASE STATES**

(75) Inventors: **Jerry L. Nadler**, La Crescenta; **Rama Natarajan**, Hacienda Heights, both of CA (US)

(73) Assignee: **City of Hope**, Duarte, CA (US)

(*) Notice: This patent issued on a continued prosecution application filed under 37 CFR 1.53(d), and is subject to the twenty year patent term provisions of 35 U.S.C. 154(a)(2).

Under 35 U.S.C. 154(b), the term of this patent shall be extended for 0 days.

(21) Appl. No.: **08/945,744**

(22) PCT Filed: **May 3, 1996**

(86) PCT No.: **PCT/US96/06328**

§ 371 Date: **Nov. 3, 1997**

§ 102(e) Date: **Nov. 3, 1997**

(87) PCT Pub. No.: **WO96/34943**

PCT Pub. Date: **Nov. 7, 1996**

Related U.S. Application Data

(63) Continuation-in-part of application No. 08/434,681, filed on May 4, 1995, now abandoned, which is a continuation-in-part of application No. PCT/US94/00089, filed on Jan. 4, 1994, which is a continuation-in-part of application No. 07/936,660, filed on Aug. 28, 1992, now abandoned.

(51) **Int. Cl.**[7] **A61K 31/195**; A61K 31/155

(52) **U.S. Cl.** **514/562**; 514/637; 514/866

(58) **Field of Search** 514/637, 562, 514/866

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(57) **ABSTRACT**

The present invention relates to a method for inhibiting the etiology of disease in patients having a disease state caused by an excess of 12-lipoxygenase or its products. In particular, the invention provides for administration of a human leukocyte 12-lipoxygenase pathway inhibitor to inhibit disease etiology, to inhibit the proliferation of breast cancer and to increase insulin receptor phosphorylation in a patient having Type II diabetics.

15 Claims, 24 Drawing Sheets

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FIG. 1A

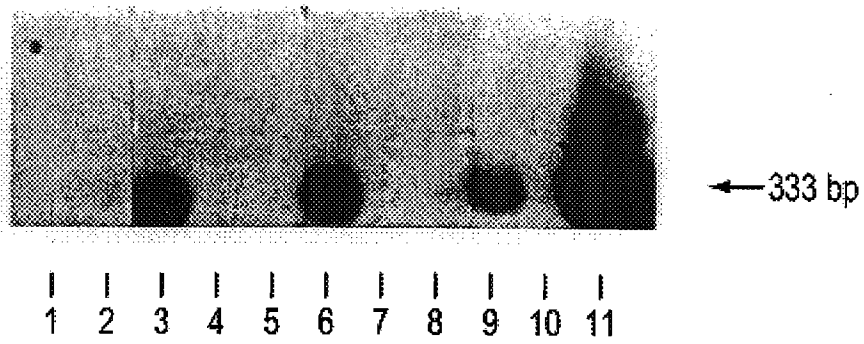


FIG. 1B

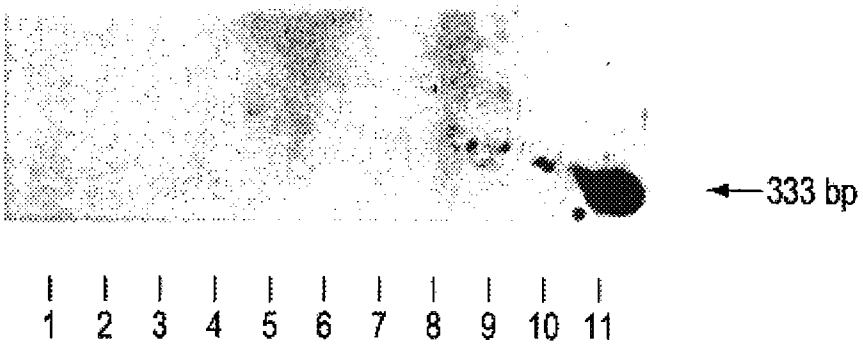
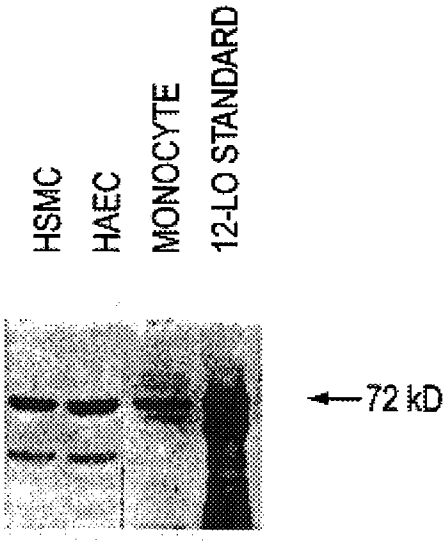


FIG. 2



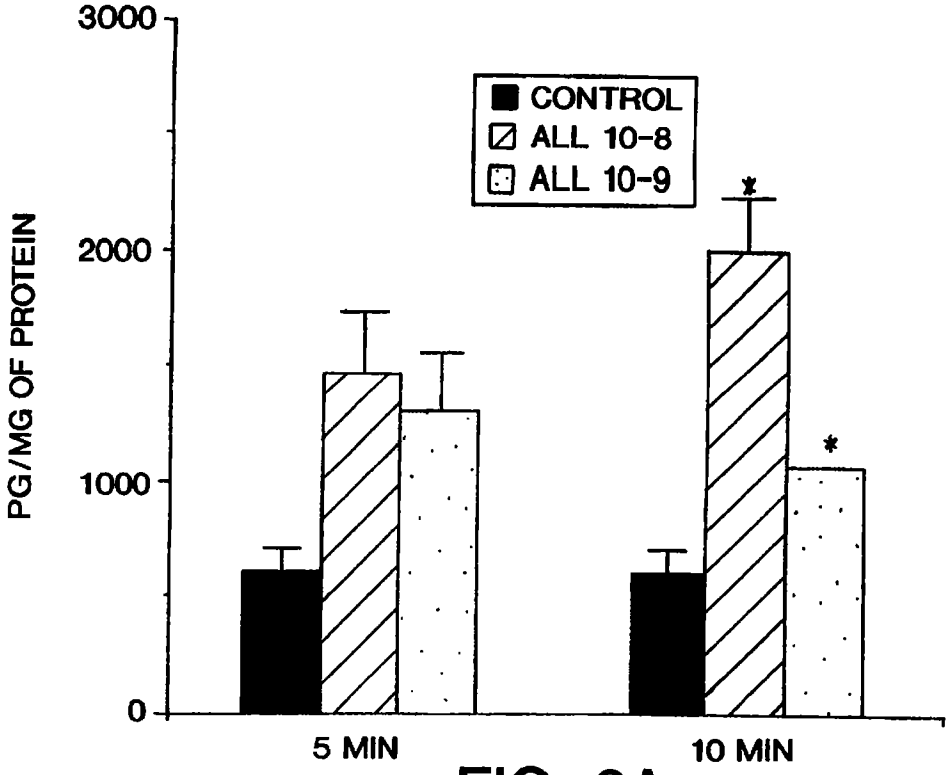

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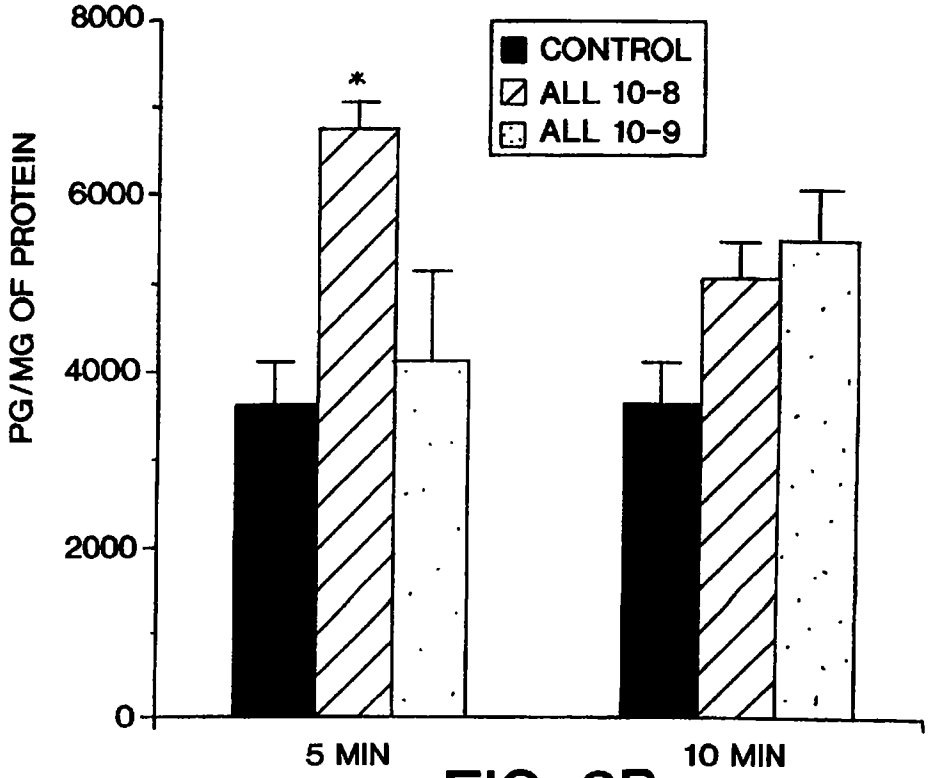
FIG. 3A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5 MIN
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FIG. 3B

FIG. 4A

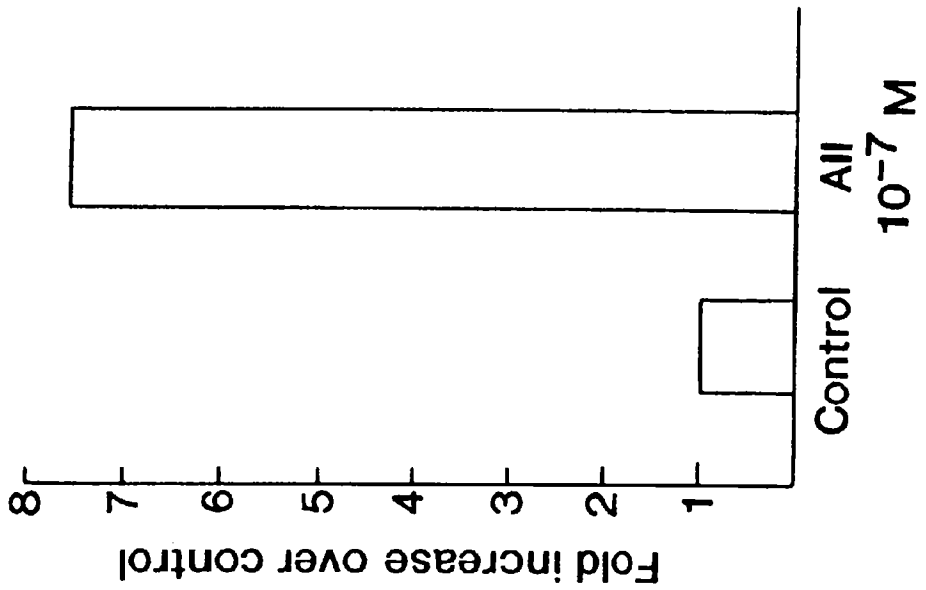

FIG. 4B

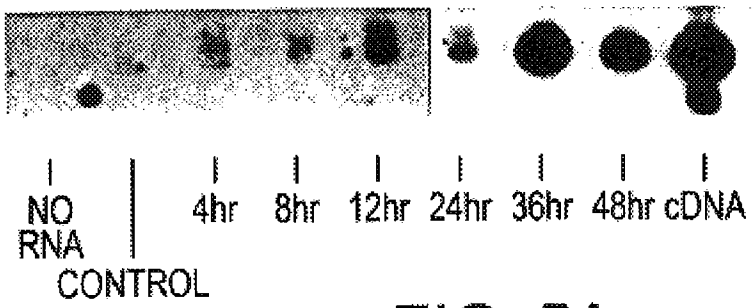
NO RNA CONTROL
4hr
8hr
12hr
24hr
36hr
48hr
cDNA

FIG. 5A

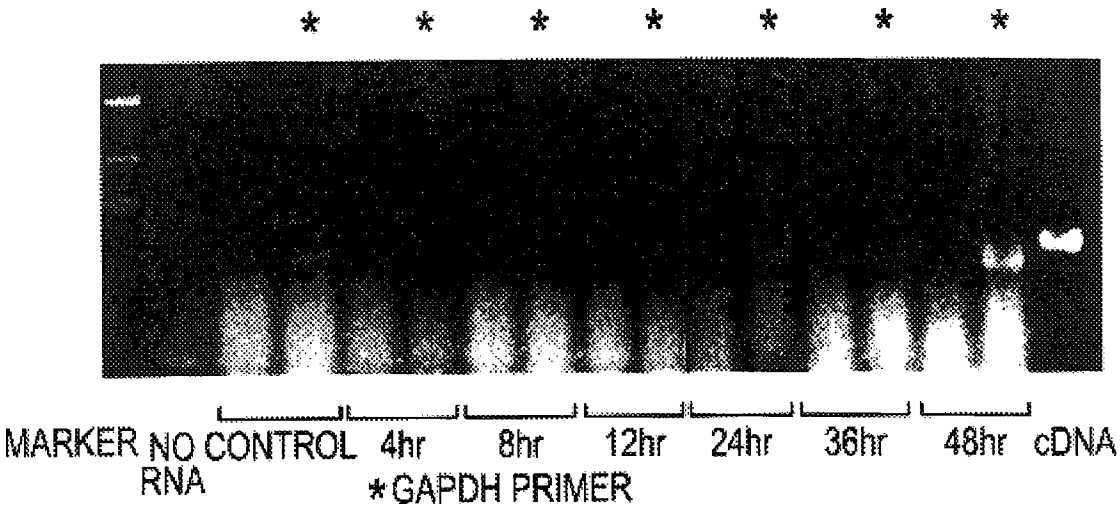
MARKER
NO RNA CONTROL
4hr
8hr
12hr
24hr
36hr
48hr
cDNA
* GAPDH PRIMER

FIG. 5B

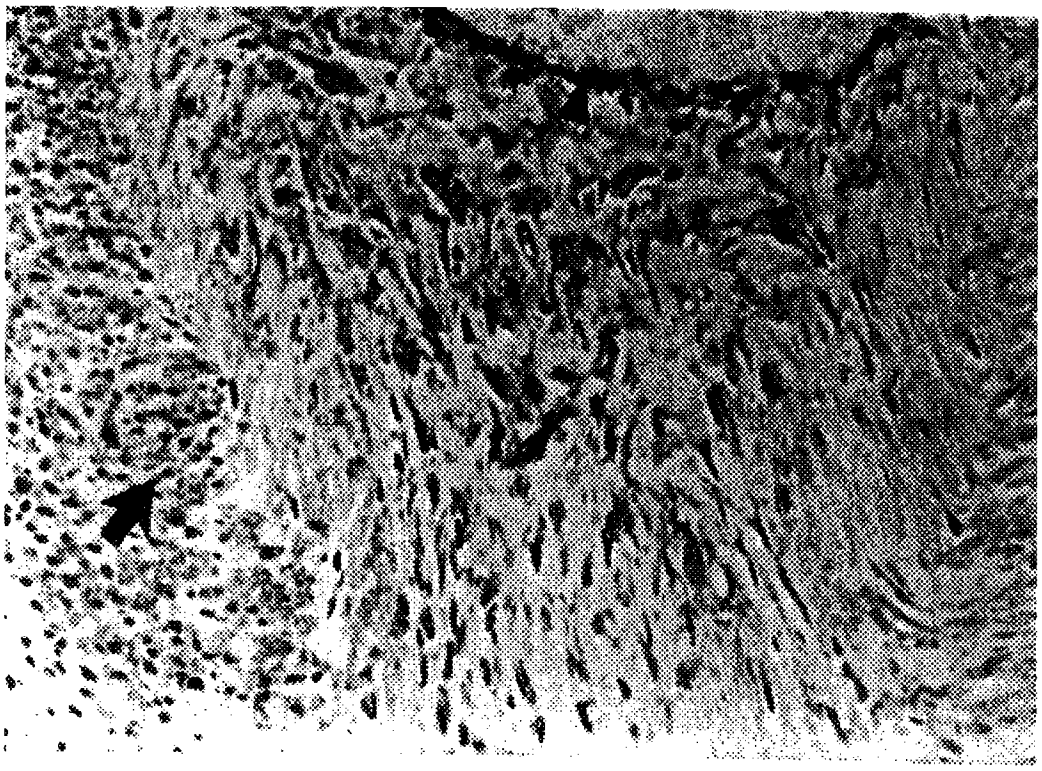

FIG. 6A

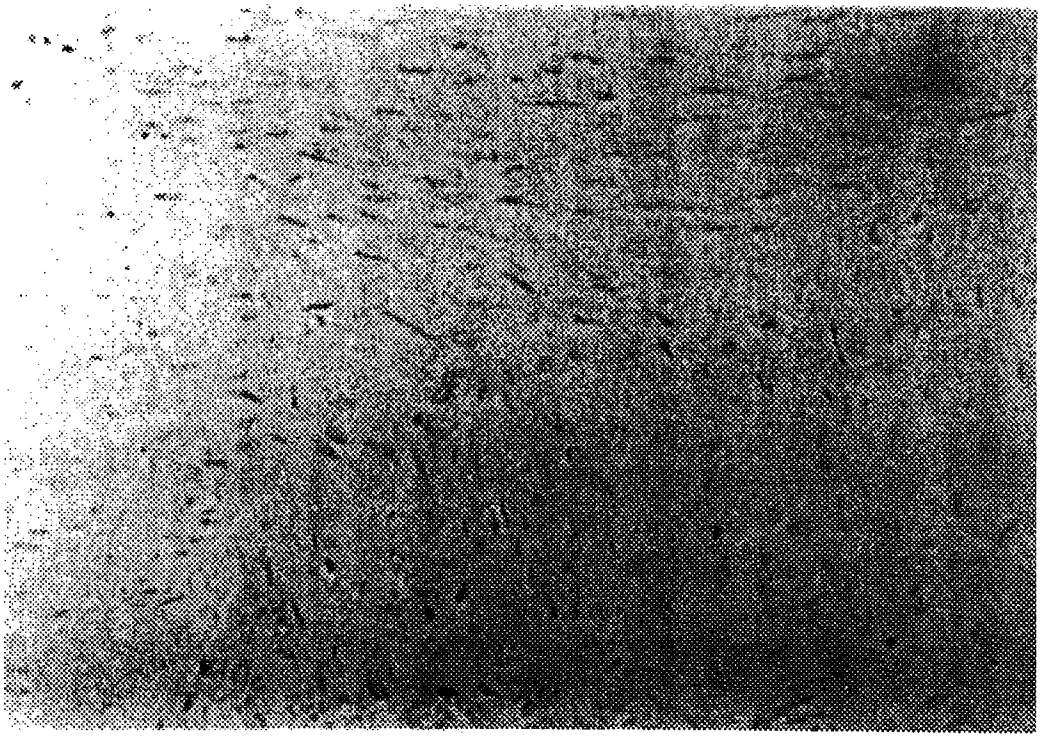

FIG. 6B

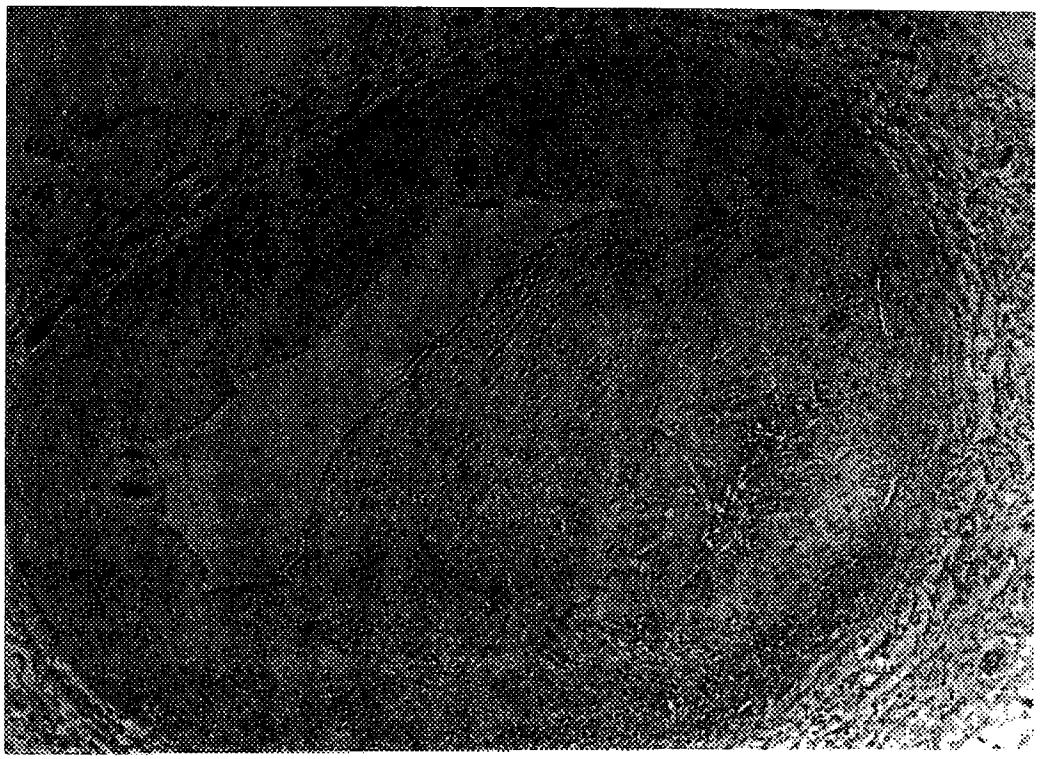

FIG. 7A

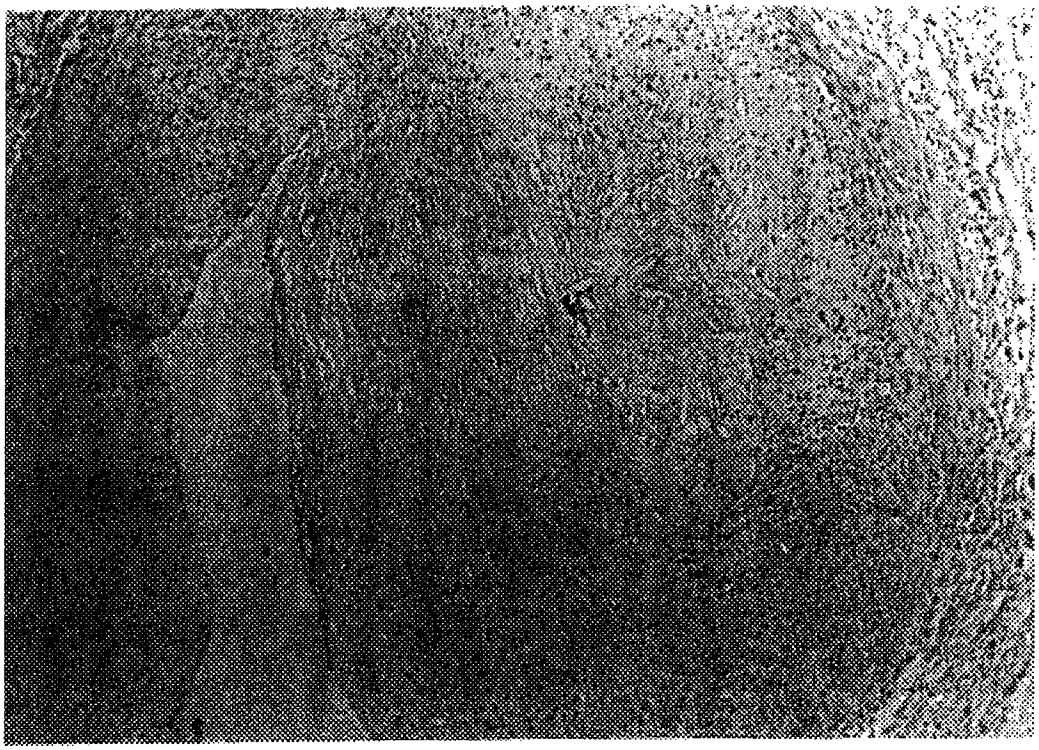

FIG. 7B

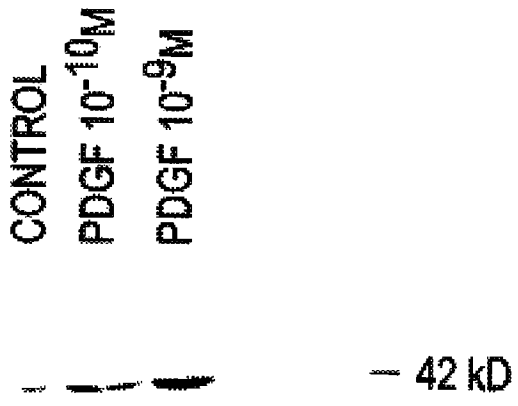
CONTROL
PDGF 10^{-10}M
PDGF 10^{-9}M
— 42 kD

FIG. 8

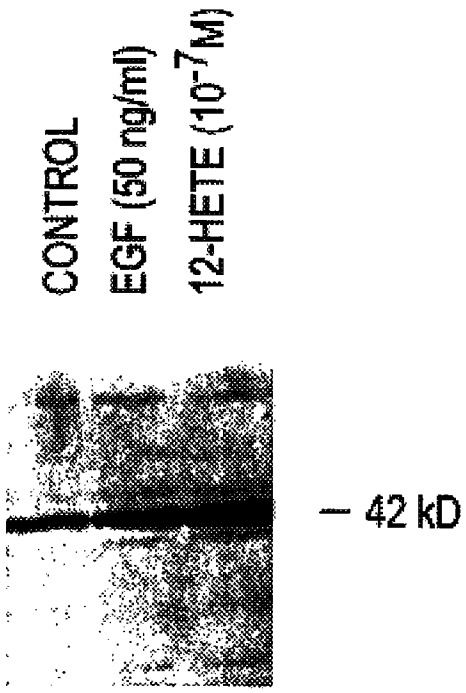
CONTROL
EGF (50 ng/ml)
12-HETE (10^{-7}M)
— 42 kD

FIG. 9

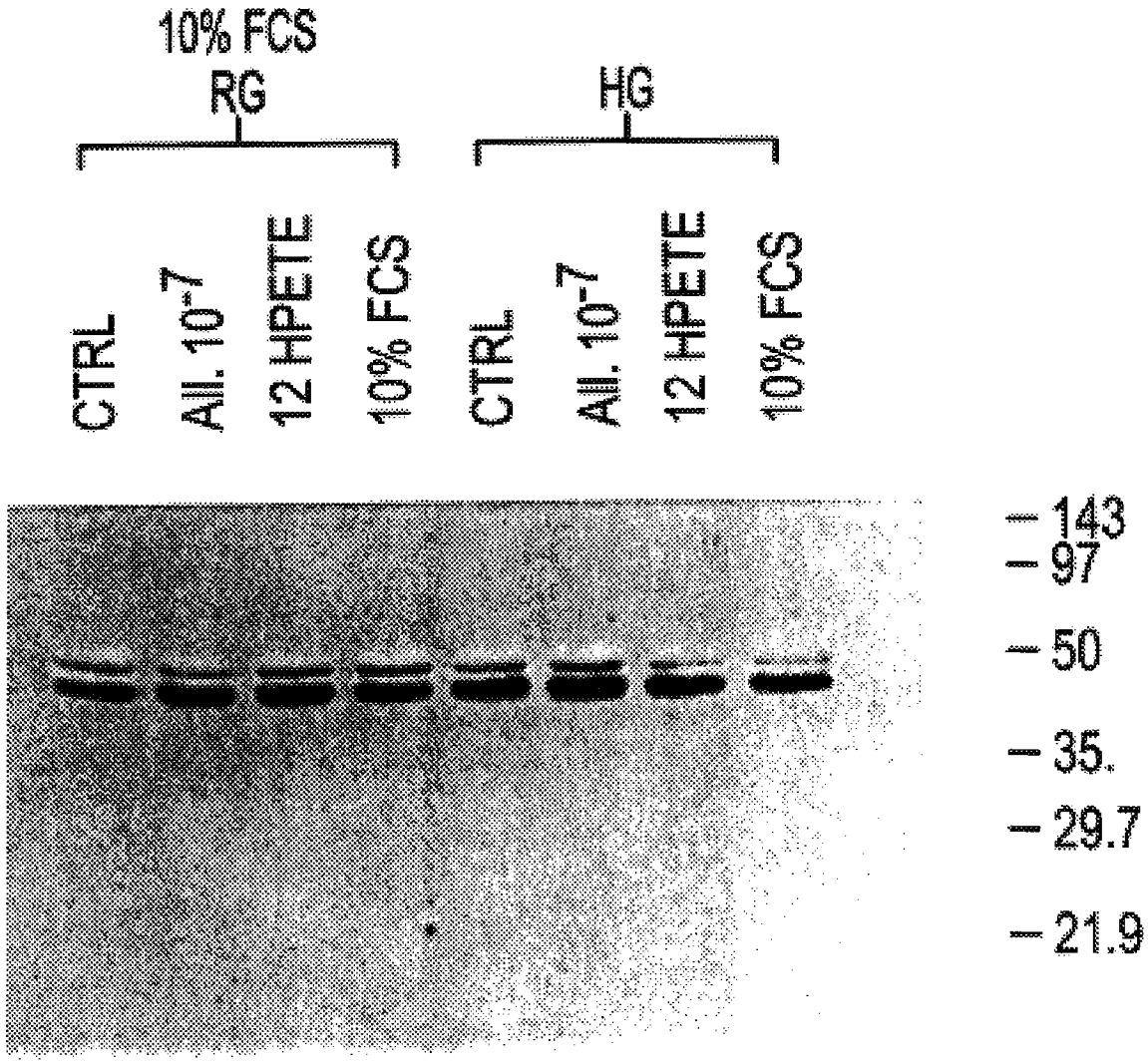
10% FCS
RG
HG
CTRL
All. 10^{-7}
12 HPETE
10% FCS
CTRL
All. 10^{-7}
12 HPETE
10% FCS
143
97
50
35.
29.7
21.9

FIG. 10

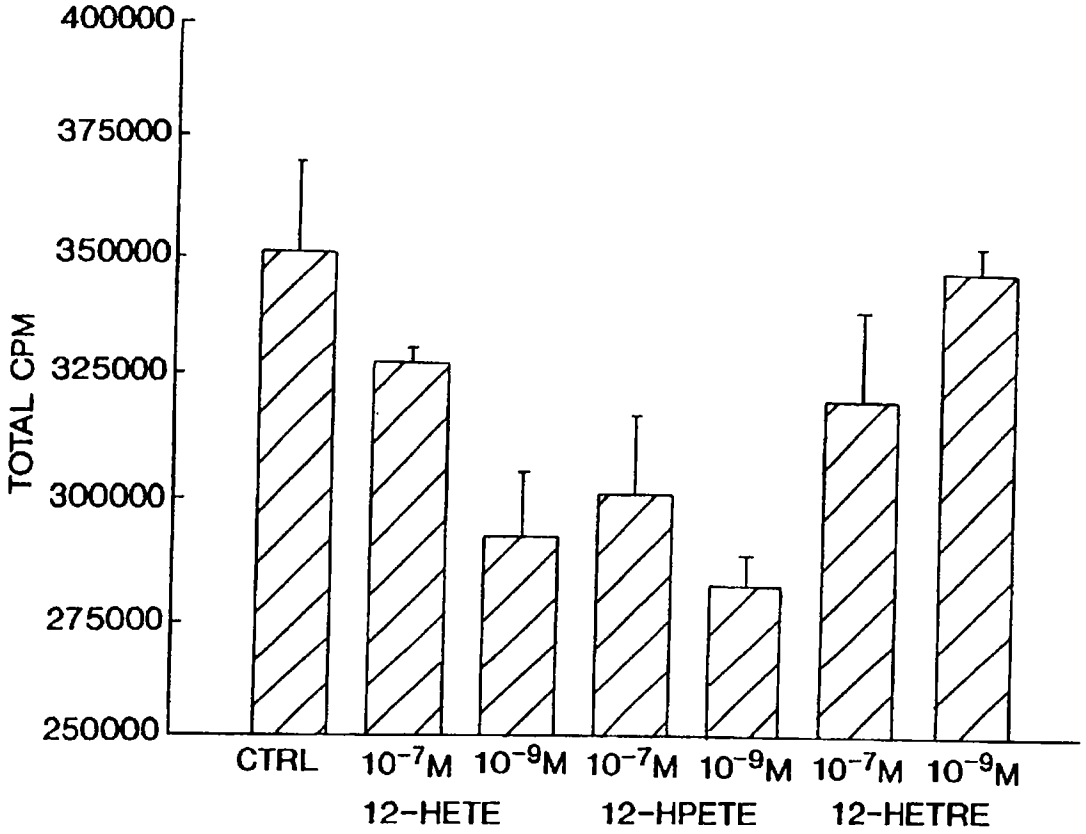
400000
375000
350000
325000
300000
275000
250000
TOTAL CPM
CTRL
10⁻⁷M
10⁻⁹M
10⁻⁷M
10⁻⁹M
10⁻⁷M
10⁻⁹M
12-HETE
12-HPETE
12-HETRE

FIG. 11

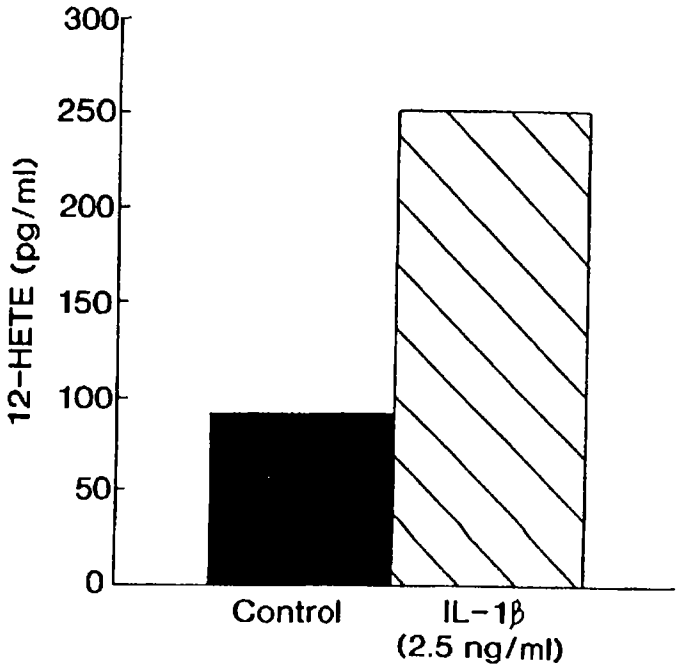
300
250
200
150
100
50
0
12-HETE (pg/ml)
Control
IL-1β
(2.5 ng/ml)

FIG. 13

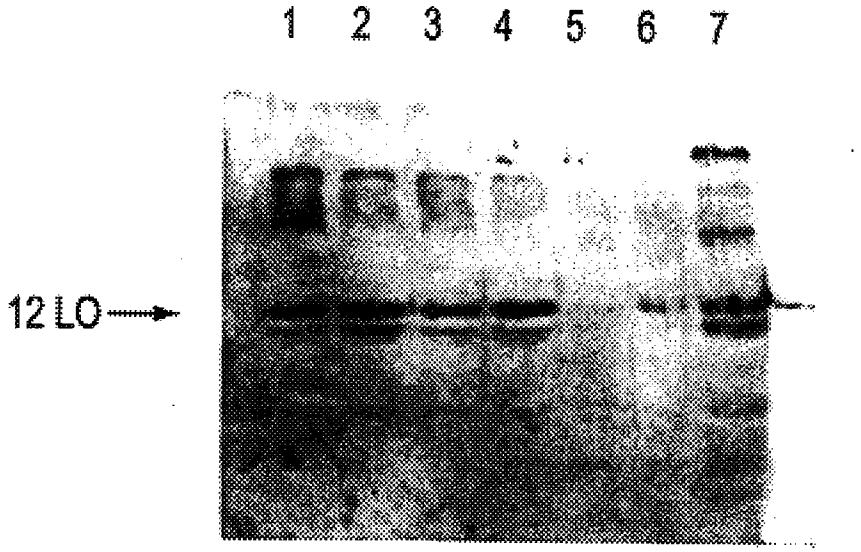

FIG. 12

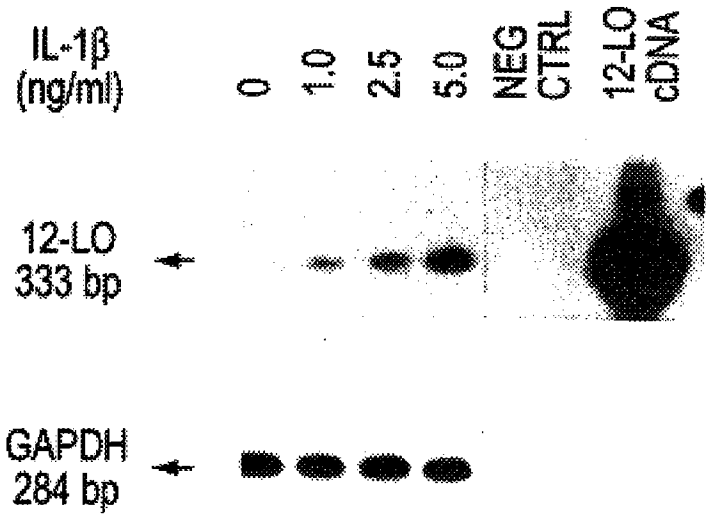

FIG. 14A

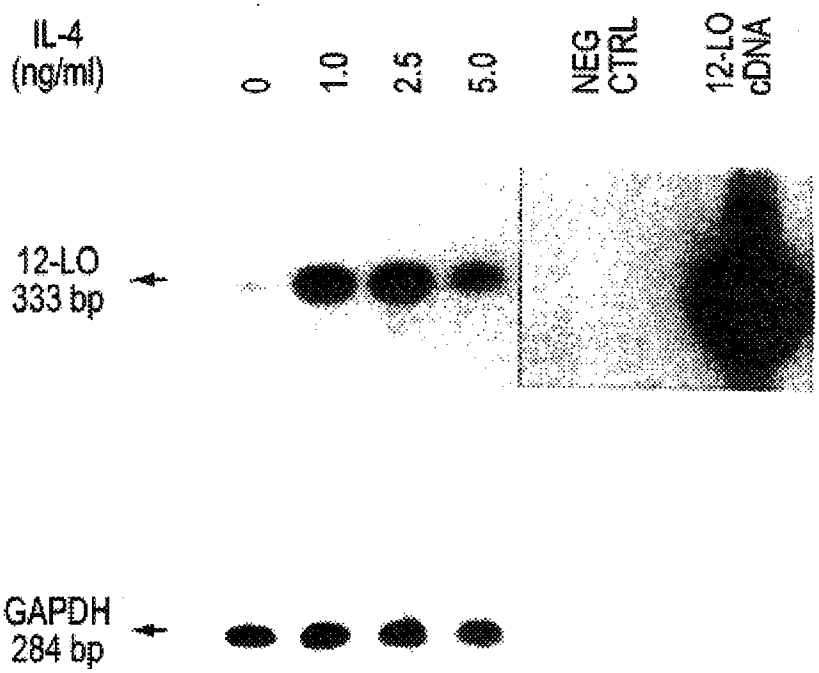

FIG. 14B

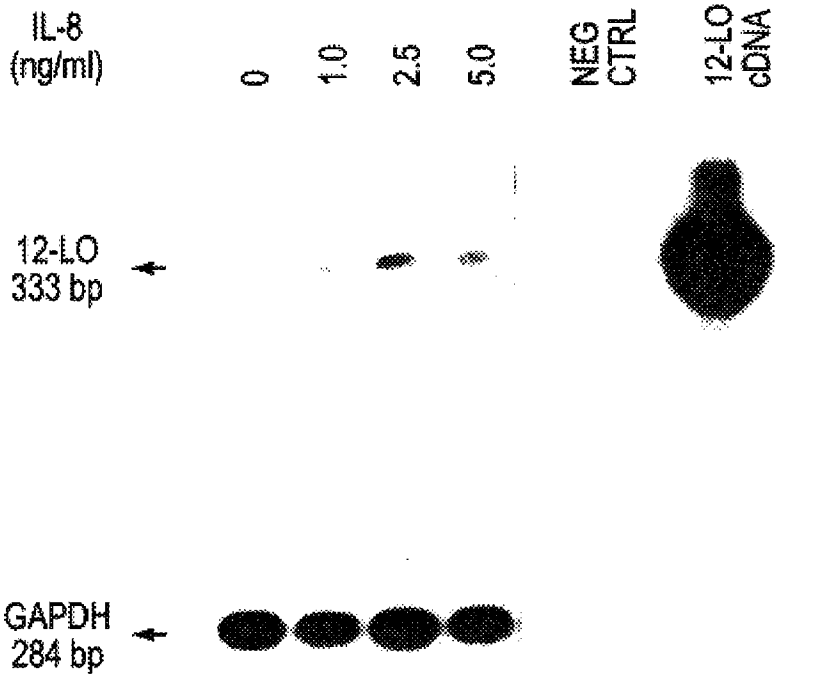

FIG. 14C

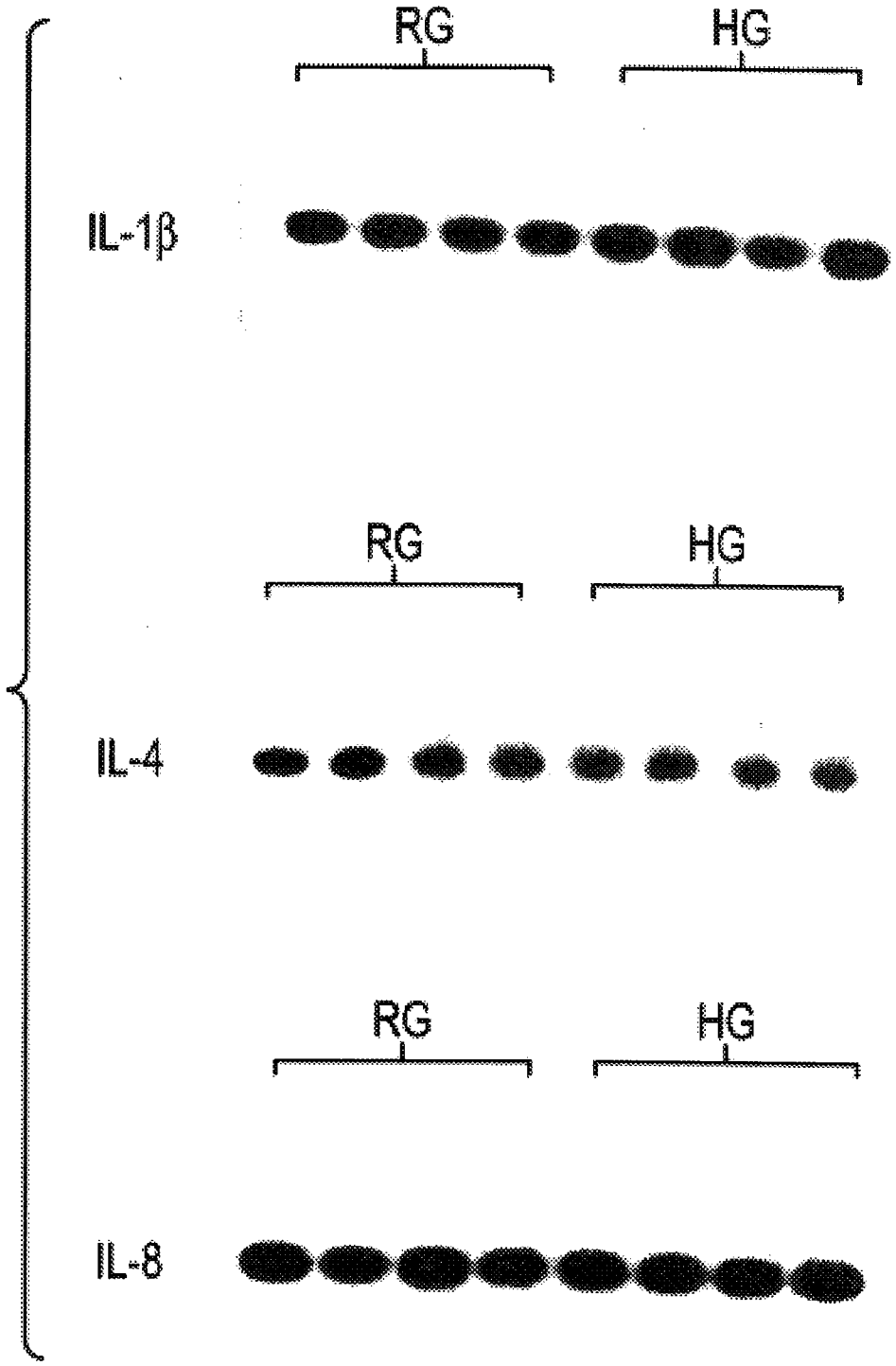

FIG. 15

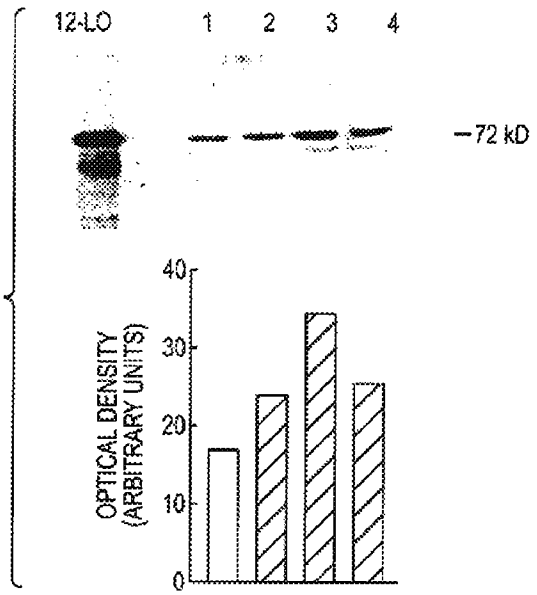
12-LO
1
2
3
4
72 kD
OPTICAL DENSITY (ARBITRARY UNITS)
40
30
20
10
0

FIG. 16

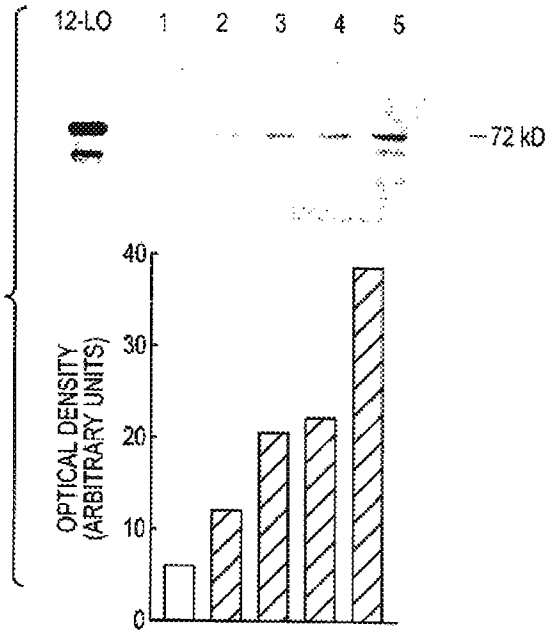
12-LO
1
2
3
4
5
72 kD
OPTICAL DENSITY (ARBITRARY UNITS)
40
30
20
10
0

FIG. 17

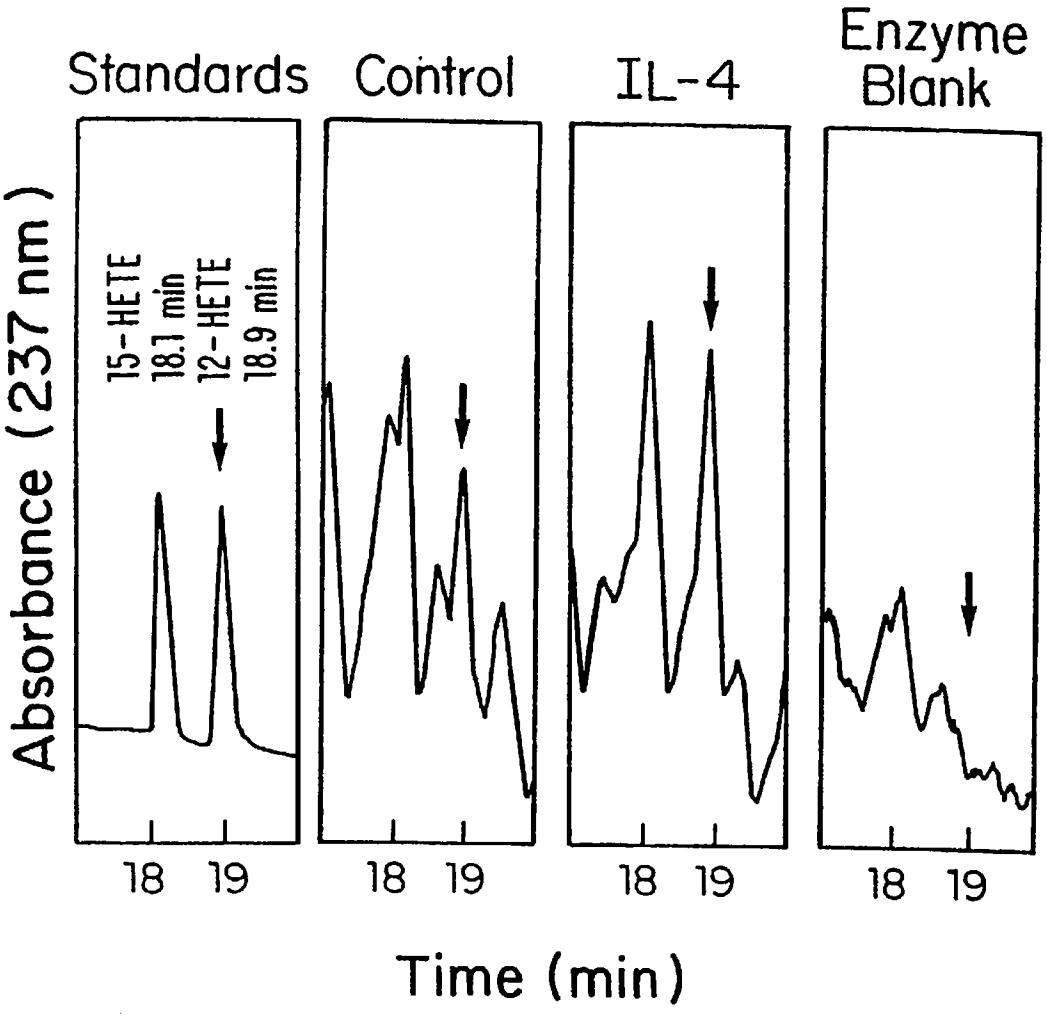
Standards
Control
IL-4
Enzyme Blank
15-HETE 18.1 min
12-HETE 18.9 min
Absorbance (237 nm)
18
19
Time (min)

FIG. 18

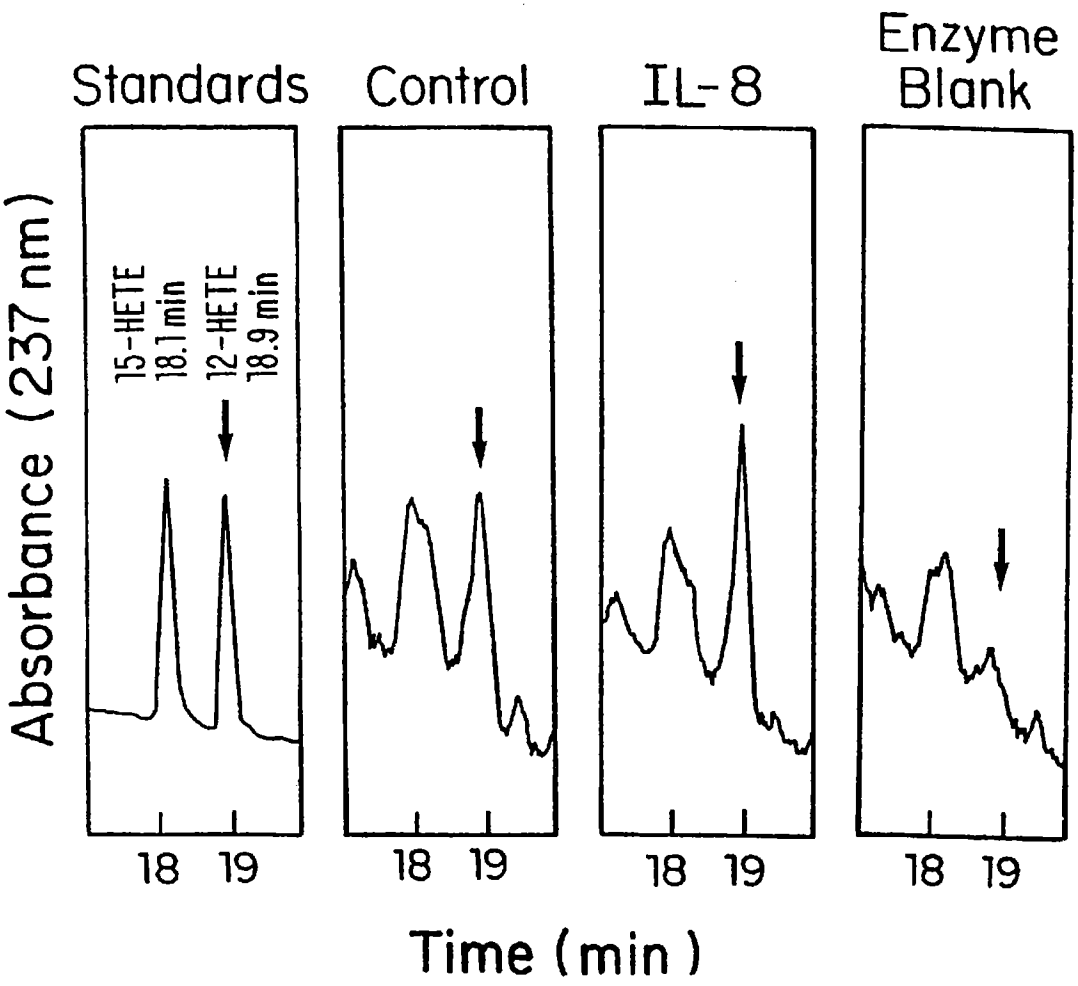
Standards
Control
IL-8
Enzyme Blank
15-HETE 18.1 min
12-HETE 18.9 min
Absorbance (237 nm)
18 19
18 19
18 19
18 19
Time (min)

FIG. 19

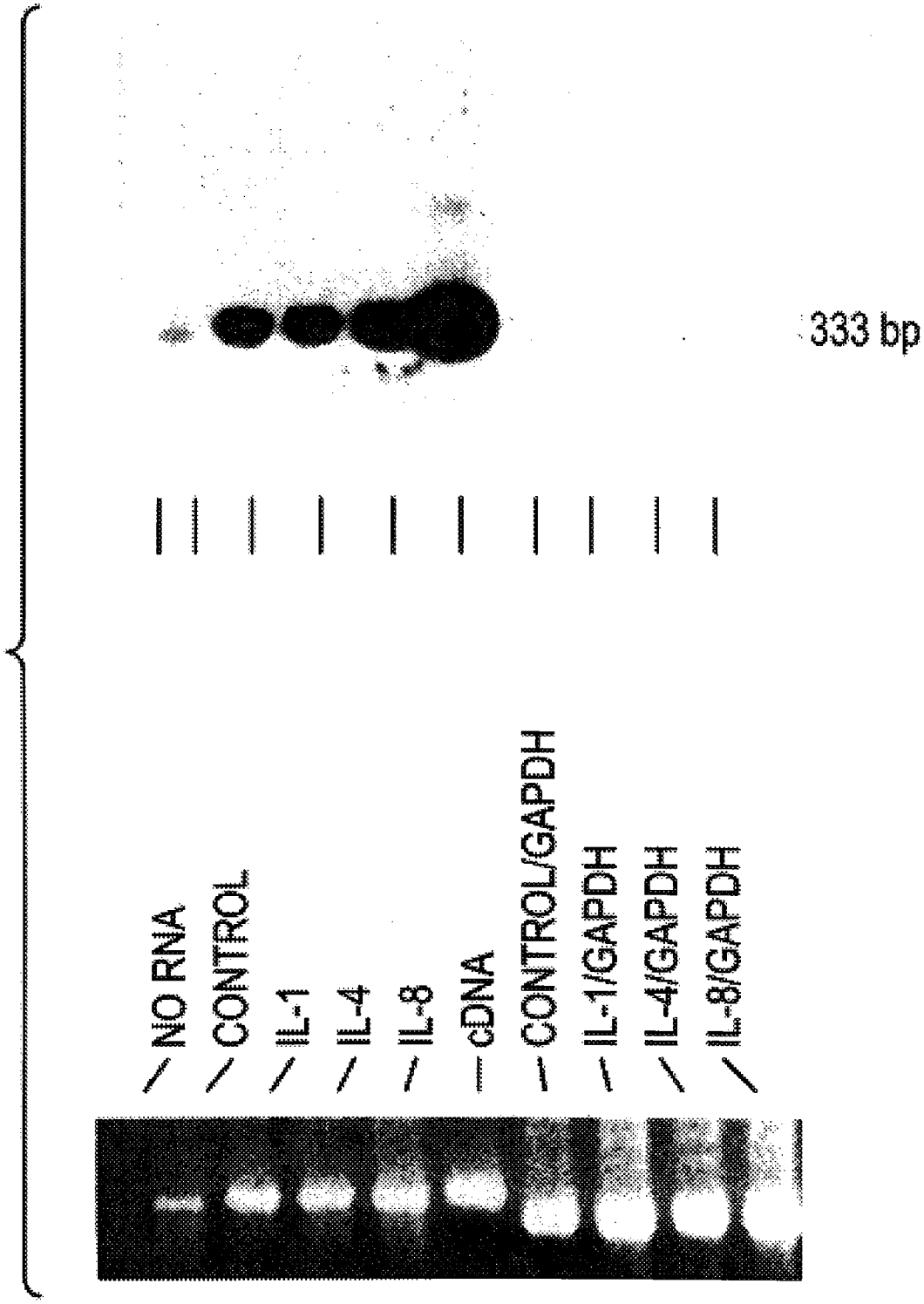
333 bp
NO RNA
CONTROL
IL-1
IL-4
IL-8
cDNA
CONTROL/GAPDH
IL-1/GAPDH
IL-4/GAPDH
IL-8/GAPDH

FIG. 20

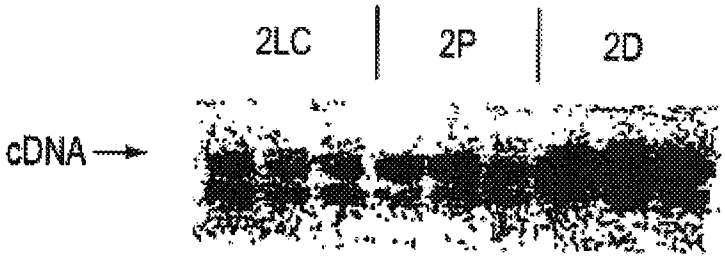
2LC
2P
2D
cDNA

FIG. 21

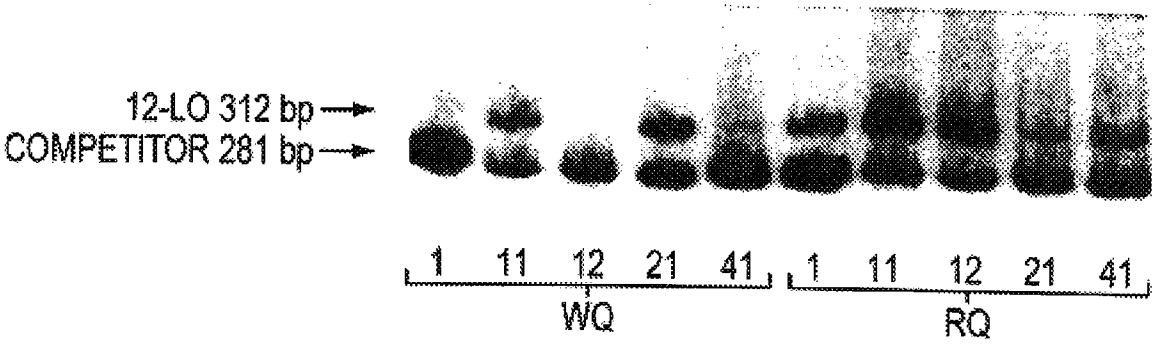
12-LO 312 bp
COMPETITOR 281 bp
1 11 12 21 41
1 11 12 21 41
WQ
RQ

FIG. 22

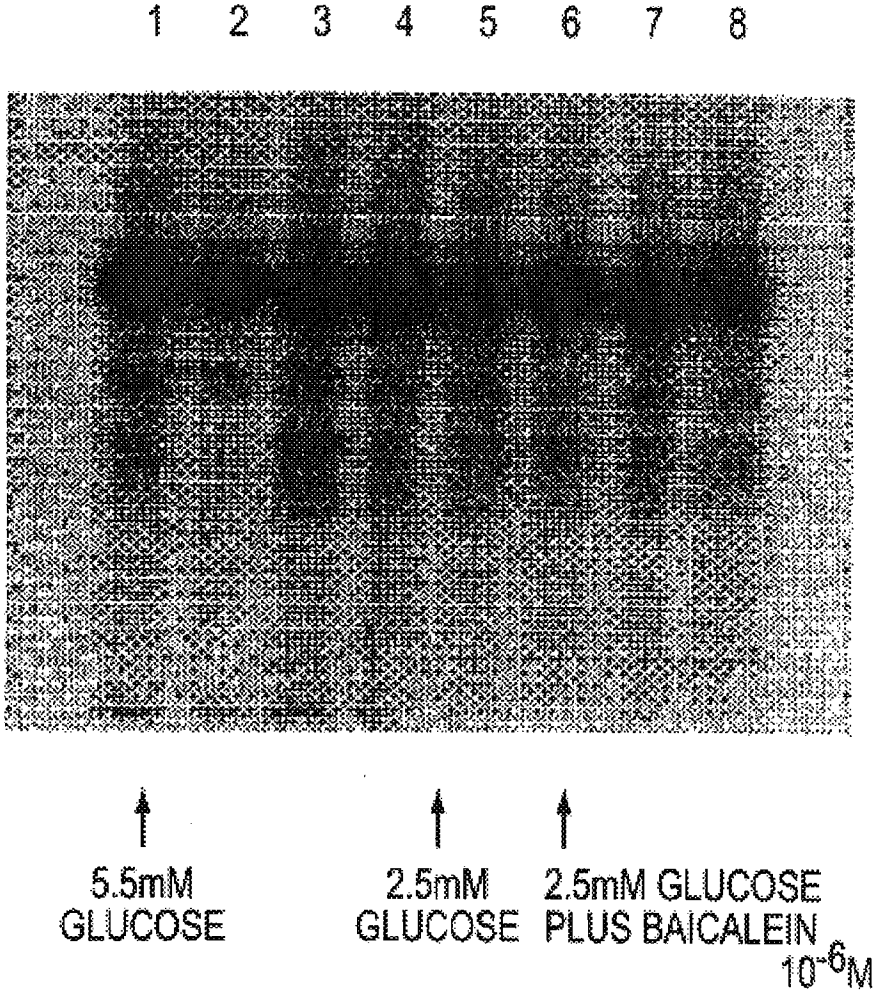
1 2 3 4 5 6 7 8
5.5mM GLUCOSE
2.5mM GLUCOSE
2.5mM GLUCOSE PLUS BAICALEIN 10^{-6}M

FIG. 23

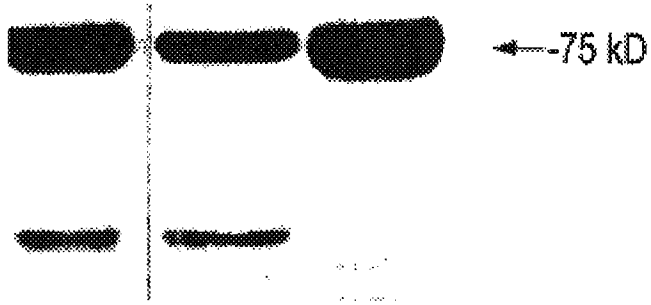
-75 kD

FIG. 26

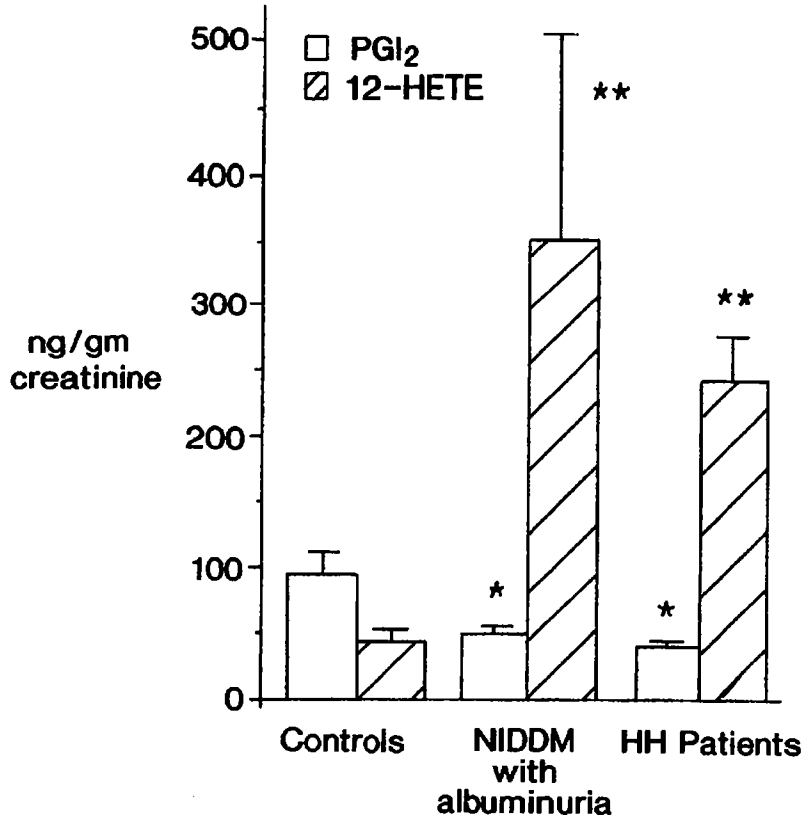
500
400
300
200
100
0
ng/gm creatinine
PGI2
12-HETE
**
**
*
*
Controls
NIDDM with albuminuria
HH Patients

FIG. 24

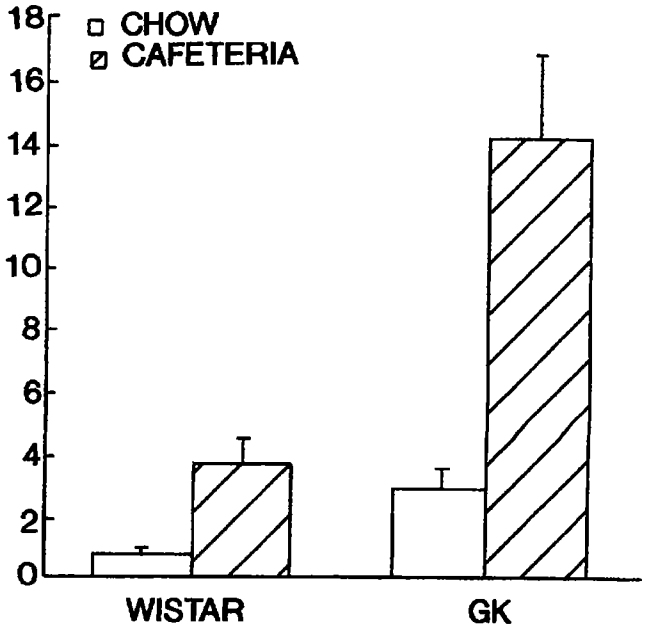
18
16
14
12
10
8
6
4
2
0
CHOW
CAFETERIA
WISTAR
GK

FIG. 25

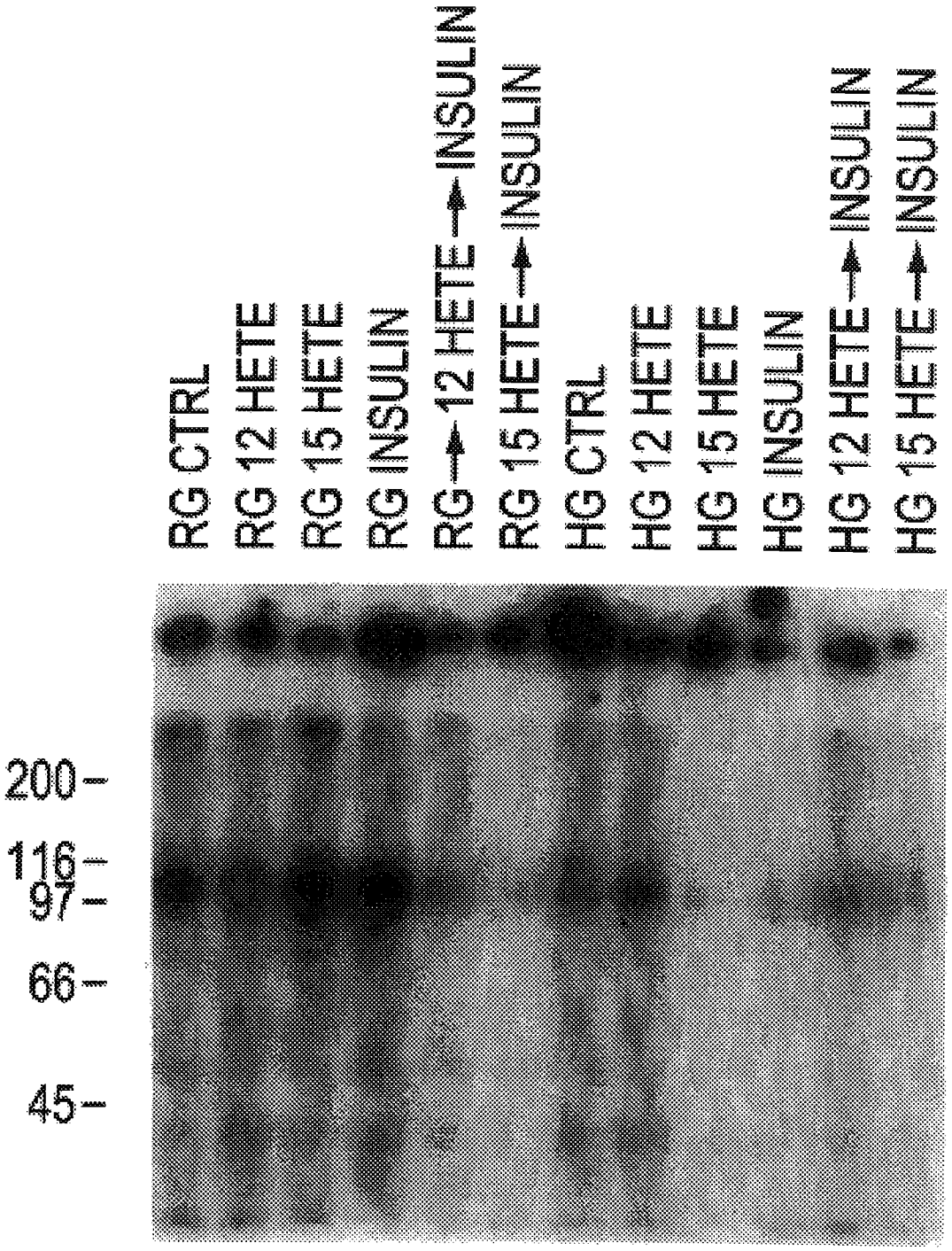
RG CTRL
RG 12 HETE
RG 15 HETE
RG INSULIN
RG → 12 HETE → INSULIN
RG 15 HETE → INSULIN
HG CTRL
HG 12 HETE
HG 15 HETE
HG INSULIN
HG 12 HETE → INSULIN
HG 15 HETE → INSULIN
200
116
97
66
45

FIG. 27

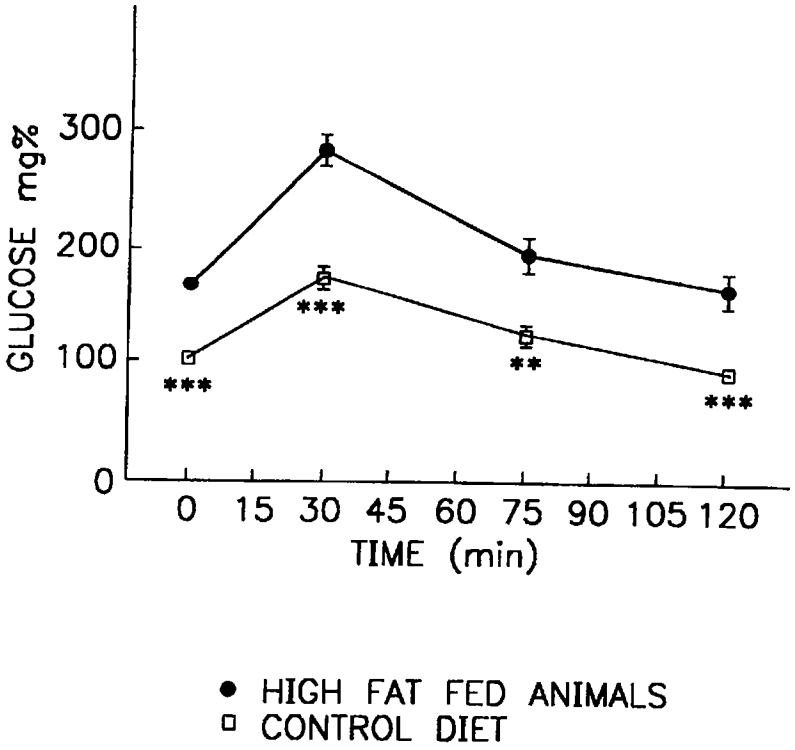

FIG. 28A

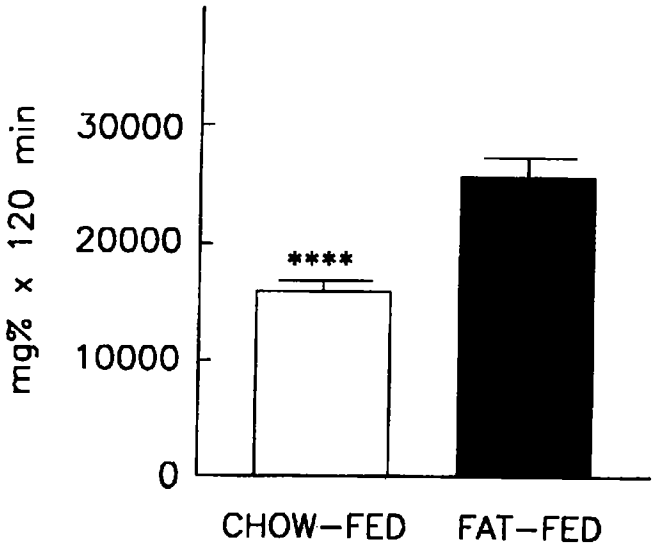

FIG. 28B

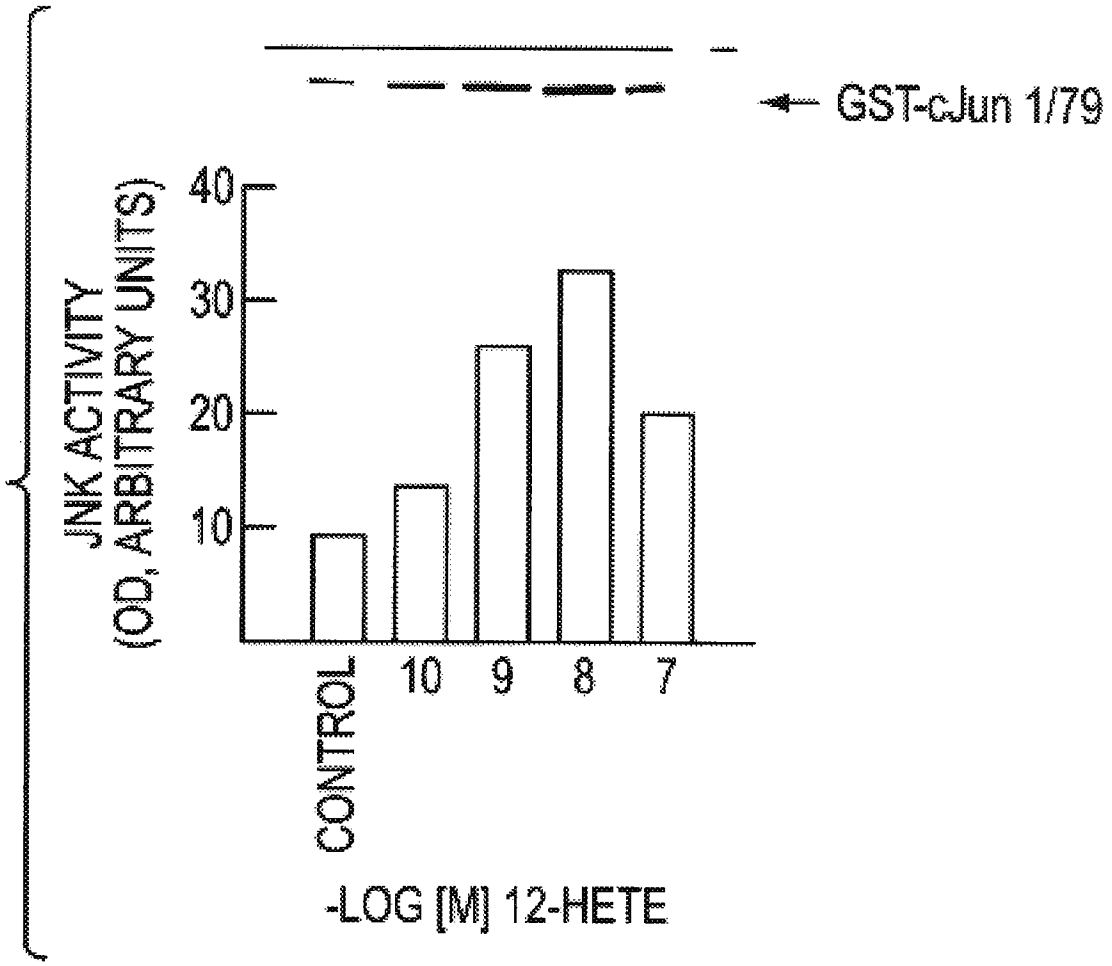
GST-cJun 1/79
JNK ACTIVITY
(OD, ARBITRARY UNITS)
40
30
20
10
CONTROL
10
9
8
7
-LOG [M] 12-HETE

FIG. 29

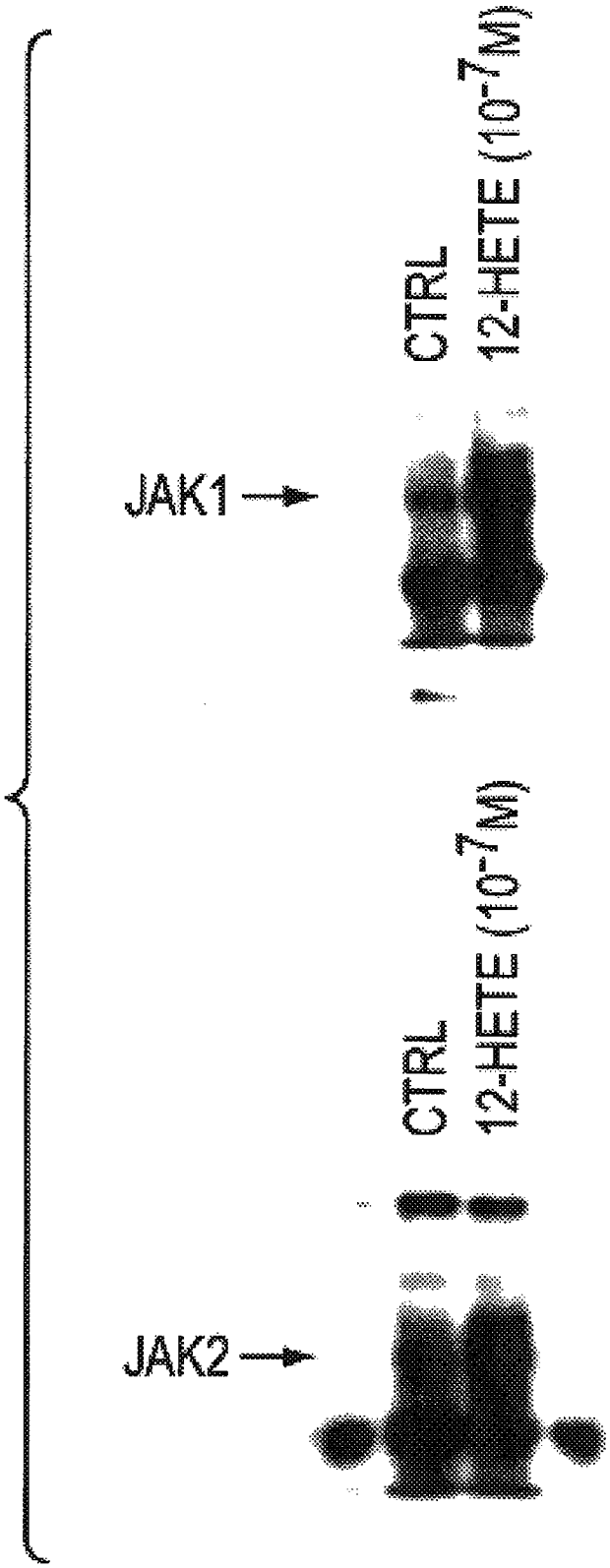

FIG. 30

HUMAN LEUKOCYTE 12-LIPOXYGENASE AND ITS ROLE IN THE PATHOGENESIS OF DISEASE STATES

RELATED APPLICATIONS

This application is a 371 of PCT/US96/06328, filed May 3, 1996, and a continuation-in-part of U.S. application Ser. No. 08/434,681, filed May 4, 1995, abandoned which is a continuation-in-part of PCT/US94/00089, filed Jan. 4, 1994, which is a continuation-in-part of U.S. application Ser. No. 07/936,660, filed Aug. 28, 1992, now abandoned.

GOVERNMENT RIGHTS STATEMENT

This invention was made with government support under Grant No. DK 39721 RO1 awarded by the National Institutes of Health. The government has certain rights in the invention.

TECHNICAL FIELD

This invention pertains to a human leucocyte type of 12-LO and its role in the pathogenesis of several major disease states.

ABBREVIATIONS

AA	=	Arachidonic acid
AII	=	Angiotensin II
EGF	=	Epidermal Growth Factor
FN	=	Fibronectin
GAPDH	=	Glyceraldehyde-3-phosphate dehydrogenase
GF	=	Growth Factor
HAEC	=	Human Aortic Endothelial Cells
HETE	=	Hydroxyeicosatetraenoic Acid
12 HETE	=	12-Hydroxyeicosatetraenoic Acid
HG	=	High Glucose
hl 12-LO	=	Human Leukocyte 12-Lipoxygenase
hl 15-LO	=	Human Leukocyte 15-Lipoxygenase
HODE	=	Hydroxyoctadecadienoic acid
12-HPETE	=	12-Hydroperoxyeicosatetraenoic Acid
HSMC	=	Human Aortic Smooth Muscle Cells
HPLC	=	High Pressure Liquid Chromatography
IL-1	=	Interleukin-1
LDL	=	Low Density Lipoprotein
LO	=	Lipoxygenase
12-LO	=	12-Lipoxygenase
15-LO	=	15-Lipoxygenase
MAPK	=	Mitogen Activated Kinase
mmLDL	=	Minimally Modified Low Density Lipoprotein
MO	=	Monocytes
NIDDM	=	Non-insulin Dependent Diabetes Mellitus
NG	=	Normal Glucose
PDGF	=	Platelet Derived Growth Factor
PKC	=	Protein Kinase C
pl 12-LO	=	Human platelet 12-Lipoxygenase
PVSMC	=	Porcine Vascular Smooth Muscle Cells
RT-PCR	=	Reverse Transcriptase Polymerase Chain Reaction
SMC	=	Smooth Muscle Cells
TGFB	=	Transforming Growth Factor Beta
TNF	=	Tumor Necrosis Factor 1
VSMC	=	Vascular Smooth Muscle Cells

BACKGROUND OF THE INVENTION

The three mammalian lipoxygenases are named according to the carbon position (**1**, **2** or **3**) at which they oxygenate arachidonic acid (**4**). There is increasing evidence that certain LO enzymes are involved in the pathogenesis and acceleration of atherosclerosis by inducing oxidation of LDL to its atherogenic form (**5,6**) and increasing the growth or migration of smooth muscle cells (**1**, **7–9**). In addition, evidence suggests that a 12-LO protein plays a role in mediating angiotensin II (AII) induced vascular and adrenal actions (**10–12**). Recent studies indicate that at least two forms of 12-LO exist, i.e., pl 12-LO cloned from human erythroleukemia cells (**2,13**) and a porcine leukocyte 12-LO which has been isolated and cloned from porcine mononuclear cells, pituitary (**14**) and bovine tracheal cells (**15**).

Applicants have demonstrated the presence of a leukocyte type of 12-LO in human adrenal glomerulose cells (**3**). The human 15-LO has been purified from human and rabbit reticulocytes (**16,17**). The human platelet and porcine leukocyte type 12-LO share 65% amino acid homology (**13**). However, porcine leukocyte type 12-LO is highly homologous to human 15-LO (86%) (**14**). Recently, it has been shown that 15-LO is expressed in macrophages of human atherosclerotic lesions but not in unstimulated monocytes (**18**).

SUMMARY OF THE INVENTION

This application describes evidence for the presence of a human leucocyte type of 12-LO enzyme (hl 12-LO) and its role in the pathogenesis of several major disease states or processes, including atherosclerosis, breast cancer, autoimmune and inflammatory disease, diabetic vascular and kidney disease and insulin resistance. There are several features of this unique enzyme that can link several seemingly diverse conditions.

1. hl 12-LO can utilize arachidonic and linoleic acid as fatty acid substrates generating hydroperoxides and other lipid mediators which can activate important signal transduction pathways commonly associated with these disorders. These mediators include (a) kinases such as specific isoforms of protein kinase C and mitogen activated kineses (MAPK), (b) transcription factors such as NFkB and oncogenes which have clearly been implicated in inflammatory and autoimmune conditions, atherosclerosis, cancer growth and metastasis.

2. Activation of the hl 12-LO enzyme can itself generate superoxide anions which can lead to the propagation of free radical processes which could accelerate the oxidative modification of lipids and proteins. These processes are involved in the pathogenesis of inflammatory, neoplastic and atherogenic conditions.

3. The hl 12-LO enzyme is strategically located. Evidence is presented showing the presence of the leucocyte type 12-LO in human monocytes, aortic vascular smooth muscle and endothelial cells, cardiac myocytes, skeletal muscle, the kidney and breast cancer cells and beta cells of pancreatic islets. These sites of activity of this enzyme allow a tissue specific role in leading to pathologic states. For instance, in the beta cells of the pancreatic islets, activation of 12-LO activity by inflammatory cytokines (e.g., IL-1) could explain the selective dysfunction and destruction of the beta or insulin producing cells of the pancreas. Furthermore, activation or increased expression of the 12-LO pathway by glucose in the beta cells could explain the dysfunctional secretion of insulin in the common form of adult diabetes (non-insulin dependent diabetes).

4. Factors increasing 12-LO expression and activity are linked to inflammatory, atherosclerotic, renal and neoplastic disease.

The factors demonstrated to increase the activity and expression of 12-LO include, (a) inflammatory cytokines associated with autoimmune disease (Type I diabetes) ath-

erosclerosis and neoplastic growth such as interleukin-1β (IL-1), (b) growth factors such as platelet derived growth factor (PDGF) and angiotensin II (AII) which have been implicated in accelerated vascular and kidney disease, and (c) hyperglycemia which has been linked to the microvascular (eye, kidney and nerve) and microvascular (heart attack, stroke and peripheral vascular disease) complications of both type I or type II diabetes.

5. Applicants have found that glucose which accounts for much of the acquired insulin resistance in diabetes increases 12-LO activity and expression in all tissues tested. Therefore, 12-LO activation could provide a common link between glucose-induced oxidative stress and development of end-organ dysfunction or damage.

Pursuant to this invention, blockade of the hl 12-LO expression or enzyme activation provides novel treatments to prevent these disease states.

IDENTIFICATION OF 12-LO IN NORMAL HSMC, HAEC AND MO

Applicants have now evaluated the precise type of LO present in unstimulated human aortic smooth muscle cells (HSMC), endothelial cells (HAEC) and monocytes (MO). Furthermore, since AII can increase the expression of 12-LO in human adrenal cells, applicants have also evaluated the effects of AII on 12-LO regulation in HSMC. Finally, applicants determined whether immunohistochemical analysis of atherosclerotic lesions demonstrates the presence of a leukocyte type of 12-LO. The results show that a 12-LO similar to that found in human adrenal glomerulose is expressed in the normal HSMC, HAEC and MO. Furthermore, this 12-LO is markedly upregulated by AII in HSMC and is present in human atherosclerotic lesions.

DESCRIPTION OF FIGS. 1 TO 7

FIGS. **1A** and **1B** illustrate RT-PCR analysis of leukocyte 12-LO RNA in HAEC, HSMC, and MO. FIG. **1A** illustrates RNA samples that were amplified for 40 cycles with leukocyte specific 12-LO primers Membranes were hybridized with porcine leukocyte 12-LO oligonucleotide probe. Lane **1** is a marker, Lanes **2, 5, 8** are negative controls without template, lane **3** represents total RNA from HAEC, with porcine leukocyte 12-LO primer, lane **4** with GAPDH primers. Lane **6** represents total RNA from HSMC with porcine leukocyte 12-LO primers, lane **7** with GAPDH primers. Lane **9** represents total RNA from MO with porcine leukocyte 12-LO primers. Lane **10** with GAPDH primers, and lane **11** is a positive control using the porcine leukocyte 12-LO cDNA.

FIG. **1B** illustrates the same RNA samples which were amplified for 40 cycles with human specific 15-LO primers. Membranes were hybridized with human 15-LO oligonucleotide. Only the 333 base pair product from amplification of the 15-LO cDNA (positive control) is shown.

FIG. **2** illustrates the expression of leukocyte 12-LO protein (72 kD) in normal HAEC, HSMC, and MO. Cytosol fractions from HAEC, HSMC, and MO were electrophoresed along with authentic porcine 12-LO protein and subjected to Western immunoblotting.

FIG. **3A** illustrates the effect of AII on 12-HETE release by HSMC. HSMC were grown to confluency. Serum was removed and cells were incubated in media **199** containing 0.4% fetal bovine serum (FBS) and 0.2% BSA for 18 hours. Cells were then washed with DME media and incubated for 20 minutes in DME media containing 0.2% BSA. AII was added to the cells for five and ten minutes at the concentrations of 10^{-9} and 10^{-8} mol/L. Media were collected for HETE assay.

*p<0.05 vs control n=4

FIG. **3B** illustrates the effect of AII on cell-associated 12-HETE levels in HSMC. After collecting supernatants, cells were washed with ice-cold PBS and harvested by scraping for the assay of cell-associated HETEs.

*p<0.02 vs control n=4

FIGS. **4A** and **4B** illustrate the regulation of leukocyte 12-LO protein expression by AII in HSMC. FIG. **4A** is an immunoblot showing regulation by AII. FIG. **4B** is a bar graph representation of densitometric analysis of immunoblot in FIG. **4A**. Cells were grown in medium 199 containing 20% FBS and serum-depleted for 24 hours by placing in medium 199 containing 0.4% FBS and 0.2% BSA. Cells were treated with AII at the concentration of 2×10^{-7} mol/L for 24 to 48 hours. Cells were washed with PBS and harvested by scraping. Cell pellets were lysed and cytosol fractions were electrophoresed.

FIGS. **5A** and **5B** illustrate the regulation of leukocyte 12-LO mRNA levels by AII in HSMC using RT-PCR. FIG. **5A** is the autoradiogram of the blot hybridized with 12-LO oligonucleotide probe. FIG. **5B** is etidium bromide stained agarose gel. Total RNA was extracted from culture HSMC incubated in low serum conditions with AII (2×10^{-7} mol/L) for different time-period shown. RNA samples were amplified for 40 cycles with leukocyte 12-LO primers or GAPDH primers.

FIGS. **6A** and **6B** illustrate microphotographs of a histologic section of an artery obtained from a below-the-knee amputation specimen from a patient with extensive arteriosclerosis. Avidin-biotin complex immunohistochemical technique was used to detect 12-LO with purified specific rabbit anti-sera. Intense staining of endothelial cells (arrowhead), cells present in endothelial thickening (small arrow) and, to a lesser degree, on the smooth muscle cells (larger arrow) were noticed in FIG. **6A**. Pre-immune rabbit sera was used at the same concentration s negative control (FIG. **6B**). (×200 magnification).

FIGS. **7A** and **7B** illustrate immunostaining of human coronary lesions using antibody to 12-lipoxygenase. Shown is a cross-section of a human left coronary artery with an advanced atherosclerotic plaque (note the cholesterol crystals in the core region) reacted with either 12-LO antibody (FIG. **7A**) or pre-immune antisera (FIG. **7B**). The darkest immunoreactivity is seen in adventitial blood vessels associated with pericytes. The medial smooth muscles cells are also immunoreactive. Lighter immunoreactivity is seen in intimal cells in plaque and non-plaque areas.

FIG. **8** is an immunoblot illustrating the dose-dependent effect of PDGF on VEGF expression in MCF-7 cells.

FIG. **9** is an immunoblot showing the effect of EGF and 12-HETE on VEGF expression in MCF-7 cells.

FIG. **10** is an immunoblot providing data on the effect of 12-HETE on VEGF expression in an immortalized human aortic smooth muscle cell line.

FIG. **11** provides data on the effects of 12-LO products on DNA synthesis in RINm5F cells.

FIG. **12** is a Western blot of proteins isolated from RINm5F cells showing the effect of IL-1β on 12-LO protein expression.

FIG. **13** illustrates the effects of IL-1β on 12-HETE production in rat islets.

FIG. **14A** shows the effects of IL-1β on 12-LO mRNA expression in porcine aortic smooth muscle cells.

FIGS. **14B** and **14C** show the same information for IL-4 and IL-8, respectively.

FIG. **15** illustrates data on mRNA for the marker GAPDH.

FIG. **16** illustrates the effect of IL-4 on leukocyte 12-LO protein expression in porcine vascular smooth muscle cells.

FIG. **17** shows the same data as FIG. **16** for IL-8.

FIG. **18** provides data on the effect of IL-4 on 12-LO activity in porcine smooth muscle cells.

FIG. **19** shows the same data as FIG. **18** for IL-8.

FIG. **20** illustrates data regarding the upregulation of human leukocyte 12-LO by IL-1, IL-4 and IL-8.

FIG. **21** shows increases in 12-LO mRNA in the pancreatic islets of increasingly diabetic rats.

FIG. **22** shows levels of 12-LO mRNA in diabetic and non-diabetic ZDF rats.

FIG. **23** present data pertaining to rat fibroblasts overexpressing the human insulin receptor at different glucose concentrations in the presence or absence of baicalein.

FIG. **24** shows data regarding the HETE/PGI_2 ratio in different diabetic groups.

FIG. **25** provides data regarding Wistar and GK rats under Chow and Cafeteria diet conditions.

FIG. **26** shows increased amounts of 12-LO in diabetic (GK) rats compared to normal (Wistar rats).

FIG. **27** shows data demonstrating that increases in phosphorylation of the insulin receptor β subunit by insulin are affected by 12-HETE.

FIG. **28A** shows glucose levels in rats fed a high fat diet versus a control diet.

FIG. **28B** represents the area under the glucose-tolerance curve in FIG. **28A** for high fat fed rats and control rats.

FIG. **29** illustrates JNK activity as a function of 12-HETE concentration.

FIG. **30** is an immunoblot showing JAK1 and JAK2 bands under control and 10^{-7}M 12-HETE conditions.

EXPERIMENTAL PROCEDURE

The lipoxygenase (LO) pathway has been implicated in leading to accelerated atherosclerosis. The precise form of 12-LO expressed in adrenal glomerulose pancreatic islets is described in application PCT/US94/00089. This application establishes that a similar precise type of hl 12-LO is present in unstimulated human aortic smooth muscle cells (HSMC), endothelial cells (HAEC) and monocytes (MO). In this study, the specific reverse-transcriptase polymerase chain reaction (RT-PCR) method was used to analyze the type of LO mRNA expressed in normal HSMC HAEC and MO. In all three cell types, a 333 base pair band was seen using primers and probes specific for the leukocyte type of 12-LO suggesting that a leukocyte type of 12-LO is expressed in these cell types. Western immunoblotting analysis in cultured HSMC, HAEC and MO using a polyclonal peptide antibody to leukocyte type of 12-LO showed a specific 72 kD band which is identical to the molecular weight of the leukocyte type of 12-LO. Angiotensin II (AII) added to normal HSMC increased 12-LO activity and expression. Immunohistochemical analysis of atherosclerotic lesions also indicated the presence of a leukocyte type of 12-LO. These results indicate that a leukocyte type of 12-LO RNA is expressed in HSMC, HAEC and MO. Also, AII upregulates 12-LO activity and expression in HSMC supporting a role for-this 12-LO pathway in human vascular disease.

1. Cells and Cultures

HAEC and HSMC were isolated from aortic specimens obtained from the heart donors in UCLA heart transplant program. HAEC at passages **5–9** and HSMC at passages **3–7** were used. HAEC were grown in medium **199** containing 20% FBS supplemented with EC growth supplement (20 mg/ml) and heparin (90 μg/ml). HAEC were identified by their typical cobblestone morphology, presence of Factor VIII-related antigen and uptake of acetylated LDL labeled with 1,1'-dioctadecyl-1-3,3,3',3'-tetramethylindocarbocyanine perchlorate (Dil-acetyl-LDL) (**19**). HSMC were grown in medium **199** containing 20% FBS and identified morphologically and immunohistochemically using HHF35, which was then visualized by a fluorescently labeled second antibody or using a biotin-streptavidin complex immunoperoxidase system (**20**). Monocytes were obtained from a large pool of healthy donors by a modification of the Recalde method (**21**).

HSMC and HAEC monolayers were washed twice with ice-cold PBS and then processed for RNA extraction or western analysis as described below. For hydroxyeicosatetraenoic acid (HETE) assay, approximately 24 hours prior to an experiment, the medium was replaced with medium **199** containing 0.4% FBS and 0.2% BSA.

2. cDNAs

Recombinant Bluescript plasmid containing the cDNA for human reticulocyte 15-LO was kindly provided by Dr. E. Sigal (Syntex Co., Palo Alto, Calif.). pUC19 plasmid containing the cDNA for porcine leukocyte 12-LO was obtained as described previously (**14**). Bluescript plasmid containing the cDNA for human platelet 12-LO was kindly provided by Prof. Bengt Samuelson (Karolinska Institute, Stockholm, Sweden) (**2**).

3. Oligonucleotide primers and probes for PCR.

$β_2$-Macroglobulin oligonucleotides were a kind gift of Dr. Perrin White (Cornell University Medical College, New York, N.Y.). Other oligonucleotides including human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) oligonucleotides were synthesized on an Applied Biosystems (Foster City, Calif.) DNA synthesizer and were purified by polyacrylamide gel electrophoresis. The sequences of oligonucleotides are listed in Table 1 and were designed based on known gene sequences (2,14,22,23) and selected from regions displaying most divergence between porcine 12-LO and 15-LO sequences (13).

TABLE 1

Primers and probes for amplification and detection

		Sequence (5'-3')	Position
Human 15-LO	5' primer	AACTCAAGGTGGAACTACCGGAG (SEQ ID NO. 1)	146–168
	3' Primer	ATATAGTITGGCCCCAGCCATATTC (SEQ ID NO. 2)	453–477

TABLE 1-continued

Primers and probes for amplification and detection

			Sequence (5'-3')	Position
		Probe	AGGCTCAGGACGCCGTTGCC (SEQ ID NO. 3)	306–326
Porcine Leukocyte 12-LO	5'	Primer	TTCAGTGTAGACGTGTCGGAG (SEQ ID NO. 4)	145–165
	3'	Primer	ATGTATGCCGGTGCTGGCTATATTT (SEQ ID NO. 5)	451–477
		Probe	TCAGGATGCGGTCGCCCTCCAC (SEQ ID NO. 6)	301–322
human GAPDH	5'	Primer	CCCATCACCATCTTCCAGGAG (SEQ ID NO. 7)	211–231
	3'	Primer	GTTCTCATGGATGACCTTGGC (SEQ ID NO. 8)	475–495
		Probe	CTAAGCAGTTGGTGGTGCAGG (SEQ ID NO. 9)	446–466
human platelet 12-LO	5'	Primer	GATGATCTACCTCCAAATATG (SEQ ID NO. 10)	472–492
	3'	Primer	CTGGCCCCAGAAGATCTGATC (SEQ ID NO. 11)	610–630
		Probe	GTTTGAGGGCCATCTCCAGAGC (SEQ ID NO. 12)	544–565

4. Amplification of Reverse Transcribed RNA Using the Polymerase Chain Reaction (RT-PCR)

Total RNA from cultured HSMC, HAEC and fresh MO was extracted with guanidiumthiocyanate-phenol-chloroform using RNAzol (Cinna/Biotecx Laboratories International Inc., Tex.). Some RNA samples were treated by RNAse-free DNAse. 3 microgram of total RNA was mixed with the PCR buffer (10 mmol/L Tris-HCl, pH 8.3, 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, and 0.001% gelatin), 200 μmol/L of each of the four deoxynucleotide triphosphates, 25 pmol each of 5'- and 3'-primers, 2 U Avian Myeloblastosis Virus reverse transcriptase (20 U/μl; Life Sciences, St. Petersburg, Fla.), and 2.5 U Taq polymerase (Perkin Elmer Cetus, Norwalk, Conn.) in a final vol of 50 μl. In some reactions, 5 pmol of each 5'- and 3'-primer of β_2 macroglobulin or GAPDH were added as an internal standard. The samples were placed in a thermal cycler at 37° C. for eight minutes for the RT reaction to proceed. Then conditions used for PCR were a denaturation step at 94° C. for one minute, annealing at 50° C. for two minutes, and extension at 72° C. for two minutes for 20–40 cycles. Blank reactions with no RNA template were carried out through the RT and PCR steps. The human 15-LO cDNA, porcine leukocyte 12-LO cDNA, and human platelet 12-LO cDNA amplifications were carried out by mixing 2–5 ng cDNA plasmid in a 50 μl vol containing 200 μmol/L of each of the four deoxynucleotide trisphosphates, 25 pmole 5'-and 3'-primers, and 2.5 U Taq polymerase. The conditions for PCR were the same as described before.

5. Gel Analysis and Blot Hybridization

20 μl aliquots of the PCR products were subjected to electrophoresis in a 1.8% agarose gel in Tris acetate-EDTA buffer. After staining with ethidium bromide and photographing, the gel was transferred onto a Zeta-probe membrane (Bio-Rad, Richmond, Calif.) by capillary blotting. The oligonucleotides used as probes were labeled at the 5'-end using [γ^{32}P]ATP and T4 polynucleotide kinase (New England Biolabs, Beverly, Mass.) and hybridized with membrane overnight in 6xSSC (1xSSC contains 0.15 mol/L NaCl, 0.015 mol/L sodium citrate), 0.5% non-fat dried milk and 7% SDS at 42° C. Membranes were washed once in 6xSSC at room temperature for 15 minutes and then once at 60° C. for 15 minutes. The washing conditions were worked out to distinguish between the PCR products of human 15-LO from those of porcine leukocyte 12-LO (**3**). The filters were exposed to Kodak x-ray film (Eastman Kodak Co., Rochester, N.Y.) with an intensifying screen at −70° C. Blots were quantitated using a computerized video densitometer.

6. Western Immunoblotting

Cells pellets were lysed in lysis buffer containing PBS (pH 7.3), 1% Triton X-100, 1 mmol/L phenylmethylsulfonyl fluoride, 50 μmol/L leupeptin, and 0.1% sodium dodecyl sulfate (SDS). Lysates were centrifuged at 10,000xg for 10 minutes. An aliquot of the supernatant (cytosol) was saved for protein estimation and the remainder saved at −70° C. for Western Blot analysis.

SDS polyacrylamide gel electrophoresis (10% running gel, 4% stacking gel) was performed according to the method of Laemmli (**24**). For Western blotting, gels were equilibrated in transfer buffer (35 mmol/L Tris base, 192 mmol/L glycine, and 20% methanol, pH 8.3) and then transferred to nitrocellulose (Hybond, Amersham, Arlington Heights, Ill.) as described by Towbin et al. (**25**), in a semidry polyblot apparatus (American Bionetics, Inc., Emeryville, Calif.) for 40 minutes. The nonspecific sites were blocked with PBS containing 10% of FCS at 4° C. overnight. The membranes were then washed twice with PBST (PBS+ 0.05% Tween-20) and incubated with primary antibody in PBST containing 1% BSA and 20% (vol/vol) FCS for 2

hours at room temperature. A polyclonal antibody against porcine 12-LO peptide with the sequence of amino acids 646–662 of the porcine leukocyte 12-LO sequence (**14**) was used. This antiserum was used at 1:100 dilution. In some studies, a polyclonal antibody against human 15-LO kindly provided by Dr. E. Sigal (Syntex Co., Palo Alto, Calif.) was used. The washed membranes were then incubated for 1 hour with second antibody (goat antirabbit) conjugated with alkaline phosphatase (1:5000; Promega, Madison, Wis.). Detection was either by color development using substrate mixture (Nitroblue tetrazoleum and 5-bromo-4-chloro-3-indolyl phosphate from Promega) or by chemiluminescence using CSPD substrate and the Western-Light Chemiluminscent detection system (Tropix, Inc., Bedford, Mass.). Nonspecific binding was evaluated using normal rabbit serum. Western blots were quantitated using a computerized video densitometer (Applied Imagining, Santa Clara, Calif.; Lynx DNA vision) and values expressed as arbitrary absorbance units.

7. Measurement of 12-LO Products

These assays were performed using previously published methods (**10,11**). Briefly, 12-and 15-HETE are extracted from supernatants and cells on C18 mini columns (Analytichem International, Calif.) and measured using our validated reverse phase gradient HPLC and (RIA) methods.

8. Measurement of Lipoxygenase Activity in HSMC

Confluent HSMC were placed in media plus 10% FCS 24 hours prior to the experiment. The cells were harvested, washed, suspended in 1 ml Tris-HCl buffer (25 mmol/L, pH 7.7) and then sonicated on ice. The assay mixture contained in 1.0 ml, 800 μl enzyme (sonicate), 100 μl $CaCl_2$ (1.5 mmol/L) and 50 μl glutathione (0.5 mmol/L). An enzyme blank was run simultaneously. The reaction was started at 37° C. with 50 μl sodium arachidonate (160 μmol/L Nu Check Prep, Elysain, Minn.) or 0.25 μCi^{14} [C] linoleic acid (New England Nuclear). After 10 minutes incubation, the reaction was stopped with 2 ml isopropanol/1.2% acetic acid followed by 2 ml chloroform. The lower organic layer was filtered and subjected to HPLC to detect HODEs or HETEs using applicants' gradient reverse phase HPLC system (**10, 11**). 12-HETE peak was identified by UV detection at 237 nM and co-migration with authentic standard (retention time 18.3 minutes). Peak heights were quantitated using a Shimazu CR5A integrator. For the identification of radioactive linoleic acid metabolites, ^{14}[C] HODEs, radioactivity in the fraction co-migrating with the same retention time as the authentic cold HODEs was quantitated. In this HPLC system, both 9- and 13-HODE have the same retention time (17.9 minutes).

9. Immunohistochemistry

The immunohistochemical method used has been previously described (**26**). Briefly, five micron sections of tissue samples derived from a lower extremity amputation for peripheral vascular disease or a coronary artery showing an atherosclerotic plaque were mounted on Silane (3-Aminoprypyltriethoxysilane, Sigma, St. Louis, Mo.) coated slides and dried overnight at 60° C. After deparaffination and dehydration they were placed in a 10 mmol/L citrate buffer solution (pH 6.0) and boiled in a microwave oven for two periods of 5 minutes each. After cooling, the sections were twice washed in distilled water. Following a 20 minute incubation in 1% hydrogen peroxide/methanol, the slides were washed twice in distilled water and twice in phosphate buffered saline (PBS). This was followed by blocking with normal horse serum 1:20 in PBS (Vector Labs). After decanting, the sections were covered with rabbit peptide anti-leukocyte 12-lipoxygenase antisera at 1:1000 dilution and incubated overnight in a humid chamber at room temperature. After two washes in PBS the slides were incubated for 40 minutes with biotinylated anti-rabbit IgG (Elite kit, Vector Labs, Burlingame, Calif.) at 1:600 dilution. After two additional washes in PBS the sections were incubated in AB complex (Elite kit, Vector Labs) at 1:200 dilution for another 40 minutes. The sections were then exposed to a Diamino benzidine solution for 7 minutes for color development. After two additional washes, the color was enhanced by incubating the sections in 1% copper sulfate for 5 minutes. All the steps were performed using an automatic stainer (Techmate 1000, Biotek Solutions, Santa Barbara, Calif.). Sections from the peripheral vessel were washed again and lightly counter stained in 6% Mayer's hematoxylin, washed, dehydrated and coverslipped. The sections form the coronary artery were not counter stained. Control slides were prepared by substituting anti-12-lipoxygenase with pre-immune rabbit serum at the same concentration.

10. Data Analysis

Immunoblots and autoradiograms were analyzed using a computer driven densitometer (Applied Imaging, Santa Clara, Calif.; Lynx DNA Vision). Data shown is representative of two to three experiments. Data generated from AII treatment of HSMC for 12-HETE synthesis was analyzed using ANOVA for multiple samples using a statistical package on a Macintosh computer system. Data is presented as mean±SE.

RESULTS OF EXPERIMENTAL PROCEDURES

Expression of a Leukocyte Type of 12-LO mRNA in HAEC, HSMC, and MO

The expression of 12-LO mRNA in HAEC, HSMC and MO was evaluated using a specific RT-PCR method since the level of detection was below the sensitivity of Northern analysis. FIG. **1**A shows expression of leukocyte 12-LO mRNA in normal HAEC, HSMC, and MO using a method highly specific for this form of 12-LO mRNA. The appropriate 333 base pair band was seen in all three cell types.

FIG. **1**B demonstrates RT-PCR analysis of human 15-LO mRNA expression from the same RNA. These results reveal no evidence for a band characteristic of human 15-LO. In a separate experiment, RNA from HAEC, HSMC, and MO was amplified and probed for the platelet type 12-LO RNA. No evidence for a human platelet 12-LO expression was found (data not shown).

Expression of hl 12-LO Protein in HAEC, HSMC and MO

To investigate whether a leukocyte type of 1–2-LO enzyme was expressed in vascular and circulating MO, the 10,000×g supernatant proteins were electrophoresed and subjected to Western analysis using a polyclonal peptide antibody derived from a sequence in the porcine leukocyte type of 12-LO that is homologous to the sequence of 12-LO found in human adrenal glomerulose. This antibody has previously been shown to lack cross reactivity to the platelet form of 12-LO and successfully demonstrated the presence of a leukocyte type 12-LO in human adrenal cells (**3**). FIG. **2** demonstrates a major 72 kD band from western analysis in HSMC, HC, and MO. Western analysis similarly performed using a polyclonal antibody directed against the human 15-LO protein did not demonstrate a band in the expected molecular weight form these cells (data not shown). HAEC

and MO produced 12-S-HETE as reflected by HPLC and RIA analysis (HAEC 2386, MO 820 pg/10^6 cells). Results for HSMC are detailed below.

Therefore, HSMC, HAEC and MO appear to express a 12-LO protein which is similar to the leukocyte type of 12-LO found in porcine tissues and human adrenal glomerulose.

Effect of AII on 12-LO Activity and Expression in HSMC and Certain Other Tissues

Another major aspect of this invention is the discovery that AII increases the activity and expression of 12-LO mRNA and protein in HSMC. FIG. **3A** shows that 5 minute incubation of HSMC with AII at the concentrations of 10^{-8} mol/L and 10^{-9} mol/L in serum free media stimulates the release of 12-HETE (control: 599±105; AII 10^{-8}M: 1467±277; AII 10^{-9} mol/L: 1296±262 pg per mg of protein). Ten minute incubations with AII also significantly simulated the release of 12-HETE at the concentration of 10^{-8} mol/L. AII significantly also increased cell-associated 12-HETE levels in HSMC (FIG. **3B**). In other studies, it was found that 12-HETE levels in response to AII could be reduced by the LO inhibitor baicalein 10^{-5} mol/L (data not shown).

To examine whether AII induces the 12-LO enzyme expression in HSMC, cells were treated with AII at the concentration of 2×10^{-7} mol/L for 24 or 48 hours. The 12-LO protein was identified by Western immunoblotting using a specific antibody to purified leukocyte type 12-LO or a peptide antibody derived from known sequences present in the human leukocyte type of 12-LO. A distinct band was detected with a molecular weight of nearly 72 kD which is the reported molecular weight of the porcine leukocyte-type of 12-LO (FIG. **4**). A 24 hour incubation of HSMC with AII in serum free media induced nearly a seven fold increase in 12-LO protein expression (FIG. **4**). In other experiments, AII was added for 48 hours also induced 12-LO expression 4–7 fold (data not shown).

In order to evaluate the specific expression and regulation of 12-LO mRNA in HSMC, applicants used a RT-PCR assay that exclusively amplifies the leucocyte type of 12-LO. The size of the PCR amplified fragment is 333 bp for both 12- and 15-LO. Therefore, specific conditions were used to distinguish leukocyte type 12-LO and human 15-LO by increasing stringency and raising washing temperature to 60° C. FIG. **5A** shows a Southern blot analysis of RT-PCR amplified products from HSMC serum-deprived for 24 hours and then treated for the indicated times with AII 10^{-7} mol/L. In this experiment, very low basal expression of 12-LO is seen. However, in other experiments in cells from various other donors, basal 12-LO expression is detectable with PCR at 20–30 cycles. AII induces 12-LO mRNA expression starting at the 12 hour incubation time and the maximum induction is shown at 36 hour incubation of cells with AII. FIG. **5B** shows the ethidium bromide stained agarose gel showing the amplification of GAPDH as an internal marker. When PCR conditions were used that were specific for either the platelet type 12-LO or human 15-LO no specific RNA band was detected (data not shown). Therefore, basal serum deprived HSMC show low expression of a leukocyte type 12-LO which is markedly upregulated by AII.

The leukocyte type of 12-LO, unlike the platelet form can also metabolize linoleic acid. Therefore, applicants evaluated whether the HSMC could form 13-HODE, the linoleic acid metabolite of LO action in addition to 12-HETE, the product of arachidonic acid metabolism. The accomplish this, applicants performed separate experiments in which the appropriate cytosolic fractions of HSM were treated with either C^{14} linoleic acid or cold arachidonic acid and the LO products of the HSMC were analyzed by a gradient reverse phase HPLC system. Cells labelled with cold arachidonic acid showed HPLC peaks co-migrating with 12-HETE. The peak height in the cell blank sample was 1.5 cm which increased to 3/5 cm in the HSMC sonicate. Cells incubated with C^{14} linoleic acid also produced 13-HODE (695 counts per minute blank to 1038 counts HSMC sonicate).

To evaluate whether a leukocyte type of 12-LO is present in atherosclerotic lesions, immunohistochemistry was performed using the peptide anti-leukocyte type 12-LO antibody. FIG. **6A** represents a high power section derived from a lower extremity amputation specimen from a patient with peripheral vascular disease. Specific cytoplasmic staining for leukocyte type of 12-LO is evident in both the endothelial and smooth muscle layers of this lesion. The neointimal area also demonstrates staining for leukocyte type 12-LO. FIG. **6B** represents a high power section from the same lesion stained with pre-immune antisera. This section demonstrates minimal background staining suggesting that the staining for leukocyte type 12-LO in FIG. **6A** is specific. FIG. **7** is a representative section from a left human coronary artery showing an advanced atherosclerotic lesion. FIG. **7A** shows definite staining for 12-LO protein in the smooth muscle and adventitial areas as well as endothelial cells. FIG. **7B** represents immunohistochemistry with the pre-immune antisera showing very little background staining with this antibody.

These results demonstrate that a 12-LO RNA and protein similar to that found in porcine leukocytes and human adrenal glomerulose (**3**) is also expressed in human vascular cells and circulating monocytes. Several approaches were utilized in this current investigation to support this conclusion. First, a peptide antibody derived from a sequence common to the porcine and human form of leukocyte type of 12-LO revealed a characteristic 72 kD band in HSMC, HAEC and MO lysates. This antibody does not cross react with the platelet form of 12-LO but has partial cross reactivity with human 15-LO (**3**). Second, a highly specific RT-PCR procedure was used to detect 12-LO mRNA in these cell types. In a previous study, it was demonstrated using this technique that a leukocyte type of 12-LO was the exclusive type of 12-LO seen in human adrenal glomerulose and U937 cells (**3**). In the present study, a specific 333 base pair amplified mRNA product was found in unstimulated HSMC, HAEC and MO when appropriate leukocyte type 12-LO primers and probe were utilized. Thirdly, in all three cell types, the 12-LO product 12-S-HETE was formed as reflected by HPLC and specific RIA. The cytosol from HSMC reacted with both arachidonic and linoleic acid to produce either 12-HETE or 13-HODE respectively. This reaction is characteristic of a leukocyte type of 12-LO and not the platelet 12-LO which only reacts with arachidonic acid to produce 12-HETE.

The human 15-LO originally cloned from the reticulocyte and found in human trachea is highly homologous (86% sequence homology) to the porcine leukocyte type of 12-LO (**14**). The PCR technique utilized here can distinguish between the leukocyte 12-LO and the 15-LO (**3**). The specificity of this approach was demonstrated using the 12-LO and 15-LO cDNA as templates for amplification (**3**). Therefore, the Southern blot hybridization using the leukocyte 12-LO probe provides the strongest evidence that the band seen reflects a 12-LO and not a 15-LO amplified product. These results are in agreement with previous stud-

ies showing no detectable 15-LO mRNA in basal or stimulated human endothelial or non-stimulated mononuclear cells (27). However, 15-LO mRNA and protein has been found in macrophage rich areas of atherosclerotic vascular lesions and in IL-4 stimulated monocytes (28) suggesting that 15-LO can play a role in advanced atherosclerotic and immune mediated vascular disease.

Increasing evidence also suggests that a 12-LO enzyme plays an important role in AII-induced actions in several additional tissues. Studies suggest that the 12-LO pathway of arachidonic acid can mediate AII-induced aldosterone synthesis in rat and human adrenal glomerulose cells (11, 12). Furthermore, recent data indicates that AII-induced adrenal cell proliferation is mediated at least in part by activation of a 12-LO enzyme. Additional studies in the rat have implicated the 12-LO pathway in the vasoconstrictive and renin-inhibitory actions of AII (29). The aorta has the capacity to produce LO products including 12 and 15-HETE (30). Recent data has revealed that both AII and high glucose can up-regulate the leukocyte type of 12-LO in cultured porcine aortic smooth muscle cells (31).

AII has major effects on vascular smooth muscle cell growth in vitro and in vivo (1,7, 32–34). In a recent report, it was found that a relatively selective 12-LO inhibitor but not a cycloxygenase inhibitor could completely prevent AII-induced hypertrophic responses in cultured porcine vascular smooth muscle cells (35). Furthermore, 12-HETE induced similar increases in protein and fibronectin content of these vascular smooth muscle cells as AII (36). The 12-LO pathway and its product 12-HETE has also been implicated in vascular smooth muscle cell migration (9). 12-HETE at concentrations as low as 10^{-12}M have been shown to lead to smooth muscle cell migration. Additional studies have demonstrated that 12-LO products can activate specific isoforms of protein kinase C and oncogenzes including ras, c-fos and jun (36–38).

Increased 12-LO activity and expression by AII may therefore be a previously unrecognized mechanism for AII-induced hypertensive and atherosclerotic vascular disease in humans. Accordingly, another important aspect of this invention entails blockade of the 12-LO pathway as a novel therapeutic modality to reduce AII related cardiovascular disease.

The 12-LO pathway in the human vascular wall and monocytes may participate in other mechanisms related to the development or progression of atherosclerotic vascular disease. Recent evidence has implicated a LO pathway in oxidative modification of LDL in the vascular wall (39–41). It is now clear that HAEC, HSMC or monocytes have the capacity to convert native LDL to minimally modified LDL which has a greater atherosclerotic potential. Of interest is the data showing that cholesterol loading of macrophages primarily leads to increased production of 12-HETE (42). A recent report has now demonstrated that both the leukocyte type of 12-LO and 15-LO can similarly oxidize lipoproteins (42). Interestingly, this same report showed a lack of ability of the platelet 12-LO to oxidize lipoproteins.

To provide additional evidence for the presence and localization of a leukocyte 12-LO in human vessels, immunohistochemical analyses of two atherosclerotic lesions were performed. The results provide immunohistochemical evidence that a leukocyte type of 12-LO is particularly expressed in the endothelial and smooth muscle cells of an atherosclerotic lesion supporting a potential role of this pathway in the early progression of atherosclerotic vascular disease. Previous studies have shown that vascular tissues and monocytes have LO activity (42–44).

Pathways by Which AII and 12-HETE Function

It has now been discovered that AII and 12-HETE effect changes in cells by stimulating mitogen activated protein (MAP) kinase activity. Specifically, AII and 12-HETE function by activating transcriptional activity of fos via ERK (extracellular regulated kinases) (e.g., ERK 1 ($P44^{MAPK}$), ERK 2 ($P42^{MAPK}$) and ERK3 ($P62^{MAPK}$)), by activating jun via JNK (cJun kinases or stress activated kinases) and/or by activating JAK (Janus kinases e.g., JAK1 and JAK2). More specifically, AII and 12-HETE activate P-21 activated kinase (PAK), which has been implicated as a key upstream signal for JNK activation.

Experimental Procedures

ERK Activity: ERK activity was evaluated by the substrate-SDS-polyacrylamide gel method described in *Anal. Biochem.,* 183:139–143 (1989). Confluent cells in 100 mM dishes were made quiescent in serum-free medium and then treated with agonists for various time periods. The cells were then lysed in lysis buffer (1% NP40, 1% sodium deoxycholate, 0.1% SDS, 100 mM NaCl, 50 mM Tris, 10 mM EDTA, 1 mM EGTA, aprotinin, 100 mg/ml leupeptin, 0.1 mM PMSF and 1 mM sodium orthovanadate, pH 7.4). Lysates were centrifuged to pellet nuclei and the cell extracts (15–20 mg protein) were subjected to electrophoresis on SDS-polyacrylamide gels (10%) containing myelin basic protein (0.5 mg/ml) as an ERK substrate. The SDS was then washed, followed by denaturation, renaturation and protein phosphorylation on the gel with [^{32}P] ATP.

JNK Activity: The plasmid pGEX-cJun 1/79 (Dept. Pharmacol. UCSD, La Jolla) is a GST-cJun (1–79) expression vector encoding amino acids (1–79) of cJun. The GST fusion protein expression vector was transformed into *E. Coli.* Protein was induced with 0.1 mM IPTG and purified by affinity binding to glutathione-agarose beads. Unstimulated or stimulated cells were lysed into WCEB (25 mM HEPEs, pH 7.7, 03.M Nacl, 15 mM $MgCl_2$, 02. mM EDTA and 0.1% triton X-100). About 50 μg protein extract was incubated with GST-cJun+GSH agarose overnight at 4° C. Phosphorylation was carried out at 30° C. for 20 minutes in the presence of 20 mM HEPES, pH 7.5, 20 mM b-glycerophosphate, 10 mM p-nitrophenolphosphate, 10 mM $MgCl_2$, 10 mM DDT, 50 μg Na_3VO_4, 20 μM ATP (cold) and about 0.5 μl of new gamma ^{32}P-ATP. After boiling with electrophoresis sample buffer, the supernatants were analyzed on 12% SDS-PAGE. JNK activity was also measured by immunoprecipitation method using an anti-JNK antibody (Parmingen Co., San Diego) and then activities were measured using 2 μg of GST-cJun (1–79) as substrate as described in *Cell,* 81:1147–1157 (1995).

PAK Activity: Cell lysis and PAK activity measurement were performed as described in *Science,* 269:221–223 (1995). Cells were lysed on ice in 50 mM Tris-HCl, 150 mM NaCl, 5 mM EGTA, 50 mM NaF, 10 mM Na pyrophosphate, 1% NP-40, 2.5% glycerol, and 1 mM Na_3VO_4 (pH 7.5), containing protease inhibitors PMSF, leupeptin and aprotinin. Cell were centrifuged for 10 minutes at 1000 g. For immunoprecipitation, the supernatants were incubated with anti-PAK1 or anti-PAK2 antibody (1:25) (Dept. Immuno. & Cell Biology, Scripps Res. Instit., La Jolla, Calif.) for 2 hours at 4° C. followed by incubation with 60 μl of 1:1 protein A beads for 60 minutes, then 5×1.0 ml lysis buffer washes and 2 washes with kinase buffer (50 mM HEPES, pH 7.5, 10 mM $MgCl_2$ 0.2 mM dithiothreitol, 2 μg myelin basic protein and 14 Mμ [r-^{32}P] ATP) for 20 minutes at 30° C. The reaction was stopped with SDS sample buffer, and results were visualized by SDS-PAGE and autoradiography.

Results

It has been discovered that the administration of AII results in biphasic activation of MAP kinase (all of the MAP kinase experiments were conducted in CHO-AT1a cells—AII receptor ATla cDNA transfected Chinese hamster ovary cells). It has been found that AII (10^{-7}M) induces a biphasic stimulation of ERK activity with a first peak of activity at 5 minutes (2–6 fold) and a later peak at 3–4.5 hours (1.5–3 fold) as well as a stimulation of JNK activity which peaks at 30 minutes and remains sustained for 1 hour. It has also been found that AII (10^{-7}M) induces stimulation of PAK with one major peak at 30 minutes (5 fold).

It has also been discovered that 12-HETE (10^{-7}M) induces a biphasic stimulation of ERK activity with a first peak at 5 minutes and a second peak at 3–4.5 hours as well as a biphasic stimulation of JNK activity with peaks at 30 minutes (2–3 fold) and 3 hours (2.5–5 fold). It has been found that 12-HETE stimulates JNK activity at concentrations as low as 10^{-9}M, as shown in FIG. **29**.

It has further been discovered that 12-HETE induces stimulation of JAK activity. CHO-AT_{1a} cells were treated with 12-HETE (10^{-7}M) for 10 hours, immunoprecipitated with a phosphotyrosine antibody and immunoblotted using specific JAK1 and JAK2 antibodies. As can be seen in FIG. **30**, 12-HETE increased JAK activity.

Finally, it has been discovered that 12-LO inhibitors (e.g., CDC and baicalein) dose-dependently reduce AII- and 12-HETE-induced mitogenic activities. Thus, the administration of a 12-LO inhibitor decreases AII-and/or 12-HETE-induced MAP kinase activity, thereby decreasing the effects which AII and/or 12-HETE have on cell growth and development.

Consistent with this data, one aspect of the present invention entails therapy for AII- or 12-HETE-induced disease in humans which includes reducing ERK, JNK and/or JAK activity via the administration of a 12-LO inhibitor. While not wishing to be bound by a particular theory, it is believed that 12-HETE increases MAP kinase activity through upregulation of 12-HETE receptors. Thus, therapy for AII- or 12-HETE-induced disease also includes reducing mitogenic activity via the administration of a 12-HETE receptor antagonist such as, for example, DuP654.

Inhibition of the 12-LO Pathway

The utilization of various pharmacologic, antisense or ribozyme methods to reduce leukocyte type 12-LO activity is described in application PCT/US94/00089. Panaxynol, a polyacetylene compound isolated from ginseng has been identified as a relatively selective inhibitor of leukocyte 12-LO (**55**) and is useful for the purpose of this invention.

Role of the 12-LO Pathway in Breast Cancer Cell Growth

Example V of application PCT/US94/00089 indicates that blockage of the 12-LO pathway provides useful human breast cancer therapy. A further evaluation of the regulation of 12-LO activity and expression in breast cancer cells and tissues confirms that proliferation of breast cancer tissue is inhibited by 12-LO inhibitors. Specifically, leukocyte-type 12-LO mRNA expression was studied by a specific reverse transcriptase PCR method in matched normal uninvolved and cancer involved breast tissue RNA samples from six patients. It was observed that in each of the six patients, the cancer involved section showed a much higher level of 12-LO mRNA (340 bp PCR product) than the corresponding normal section (3–6 fold higher after normalization to the internal control for PCR, GADPH mRNA 284 bp). 12-LO mRNA levels were also 7- and 11-fold greater in two breast cancer cell lines, MCF-7 and COH-BR1 compared to the normal breast epithelial cell line, MCF-10F. In addition, the proliferation of MCF-7 cells was significantly inhibited by three LO inhibitors, baicalein (10 μM), CDC (10^{-5}M) and NDGA (30 μM), but not by a cyclooxygenase inhibitor, ibuprofen (10^{-5}M). Treatment of serum-starved MCF-7 cells with EGF for four hours lead to a dose dependent increase in the formation of the 12-LO product, 12-HETE (basal 257 ±10 pg/10^6 cells; EGF 50 ng/ml 462±15 pg; EGF 100 ng/ml 593±46 pg, both $p<0.001$ vs basal). EGF (50 ng/ml) also led to a marked increase in the levels of the 12-LO protein (72 kD) as well as 12-LO mRNA at 24 hours. Hence, activation of the 12-LO pathway appears to play a key role in basal and EGF-induced breast cancer cell growth and development.

Role of the 12-LO Pathway in the Action of Estrogen in Breast Cancer

It has now been discovered that estrogen, which has been linked to breast cancer cell growth and development, plays a role in activating the 12-LO pathway in breast cancer cells.

Treatment of cells from the estrogen receptor positive breast cancer cell line, MCF-7, with 17β-estradiol for 4 hours in a defined serum-free and phenol red-free medium led to a dose-dependent increase in the levels of cell associated 12-LO product 12-hydroxyeicosatetraenoic acid (12-HETE) (Basal 161±29 pg/10^6 cells; 17β-estradiol, 5 nM 784±150 pg; 17β-estradiol, 10 nM 1056±187 pg; both $p<0.001$ vs basal). This stimulatory effect of 17β-estradiol on 12-HETE was not observed in the estrogen receptor negative cell line MDA-MB-231. Treatment of MCF-7 cells with estrogen for 22 hours also caused a dose-dependent increase in the expression of the leukocyte-type 12-LO protein as examined by immunoblotting with a 12-LO peptide antibody (2.1-fold and 3-fold increase over basal at 1 nM and 10 nM 17β-Estradiol respectively). Thus, 17β-estradiol increased 12-LO activity and expression in MCF-7 cells. Hence, activation of the 12-LO pathway appears to play a key role in estrogen-induced breast cancer cell growth and development.

Consistent with this data, one aspect of this invention entails therapy to reduce breast cancer cell growth and development through inhibition of the 12-LO pathway. Such 12-LO pathway inhibition would, inter alia, reduce the effect estrogen has on breast cancer cell growth and development.

Role of the 12-LO Pathway in the Formation of VEGF

Applicants have also discovered that the 12-LO pathway plays a role in the formation of vascular endothelial growth factor (VEGF). VEGF is an endothelial cell-specific mitogen which increases vascular permeability and monocyte migration. VEGF appears to be a major angiogenic factor for many types of cancer, including breast and lung cancer. Further VEGF has been linked to the development of proliferative diabetic retinopathy as well as accelerated vascular disease often associated with diabetes.

DESCRIPTION OF FIGS. **8**, **9** AND **10**

FIG. **8** illustrates the dose-dependent effect of platelet-derived growth factor (PDGF) on vascular endothelial growth factor (VEGF) protein (42 kD) expression in MCF-7 breast cancer cells. Nearly confluent MCF-7 cells were

serum starved for 24 hours by placing in DME medium+ 0.4% FCS and 0.2% BSA. This medium was then freshly replaced and the cells incubated for another 24 hours with PDGF. At the end of the incubation, the cell monolayers were washed with ice-cold PBS, scraped into PBS and pelleted by centrifugation. The cell pellets were then lysed and equal amounts of protein (50 μg) subjected to electrophoresis and immunoblotting to detect VEGF using a specific antibody from Santa Cruz Biotechnology. Detection was by a chemiluminiscent technique. It is clearly seen that PDGF causes a dose-dependent increase in the expression of VEGF in the breast cancer cells.

FIG. **9** illustrates the effect of epidermal growth factor (EGF) and the 12-lipoxygenase product 12-HETE on VEGF protein expression in the MCF-7 breast cancer cell line MCF-7. MCF-7 cells were treated with EGF and 12-HETE for 24 hours and VEGF protein identified as described in the legend to FIG. **8**. The figure shows that VEGF protein expression is not only induced by a breast cell mitogen such as EGF but also by the 12-LO product, 12-HETE. In fact, 12-HETE appears to be more potent than EGF in inducing VEGF indicating that 12-HETE has potent angiogenic properties.

FIG. **10** illustrates the effect of a 12-lipoxygenase product 12-HPETE on VEGF protein (42 kD) expression in an immortalized human aortic smooth muscle cell line (AIHSMC). The cells were serum starved for 24 hours and then placed in fresh medium along with 12-HPETE. Cells were incubated for five hours and VEGF protein was then identified in cell lysed as described in the legend to FIG. **8**. Lane **3** shows that the 12-LO product, 12-HPETE causes an increase in the expression of the angiogenic agent, VEGF, in PVSMC when compared to the control in lane **1** as indicated in the figure.

FIGS. **8** and **9** report data in two human breast cancer cell lines MCF-7 and MDA MB that show that the 12-LO product 12-HETE at 10^{-7}M and 10^{-8}M increases VEGF protein expression. FIG. **10** shows that 12-HETE can increase VEGF protein in HVSMC.

In addition to the work reflected in FIGS. **8–10**, it has now been discovered that AII, 12-HETE and hyperglycemia (HG) increase VEGF production in vascular smooth muscle cells (VSMC). Porcine and human VSMC were cultured for at least two passages under normal glucose (NG, 5.5 mM) or under HG conditions. VEGF protein expression was determined by Western blotting and VEGF mRNA by Northern blots. HG alone increased the level of VEGF mRNA (2.8-fold) and protein (3.2-fold). In addition, VEGF protein (45 K) and mRNA (3.7 Kb) expression were markedly increased by 4 hours of treatment with AII (10^{-7}M) in the cells cultured in HG (2.2- and 1.4-fold resp.). Furthermore, 12-HETE (10^{-8}M and 10^{-7}M for 4 hours) increased the expression of both VEGF protein and mRNA in cells cultured under both NG as well as HG conditions. In addition, HG increased the secretion of VEGF into the medium as measured by a specific EIA (56.2±4 ng/ml NG vs 73±5 HG, pc<0.02).

AII and 12-HETE also increased VEGF secretion by 1.24 and 1.4-fold, respectively, as measured by EIA.

Consistent with this data, one aspect of this invention entails therapy to reduce breast and other cancer metastic potential as well as afflictions associated with diabetes (e.g., proliferative diabetic retinopathy and accelerated vascular disease) by reducing VEGF production, for example, through inhibition of the hl 12-LO pathway.

Role of the 12-LO Pathway in the Pathogenesis of Type I Diabetes

This aspect of this invention involves the role of 12-LO pathway activation in the pancreatic beta cell dysfunction or cytotoxicity in response to cytokines implicated in the pathogenesis of type I diabetes.

DESCRIPTION OF FIGS. 11, 12 AND 13

FIG. **11** illustrates the effects of 12-LO products on DNA synthesis in the insulin-producing rat beta cell line, RINm5F. DNA synthesis was studied using ^{3}H-thymidine incorporation (luCi/ml) added for the last six hours (18–24 hours of experiment). The LO products were added to the cells in complete growth media for 24 hours. As demonstrated, 12-hydroxyeicosatetraenoic acid (12-HETE, 10^{-9}M) and 12-hydroperoxyeicosatetraenoic acid (12-HPETE) reduced ^{3}H-thymidine incorporation reflecting their effects to decrease DNA synthesis in this beta cell line.

FIG. **12** illustrates a western immunoblot of proteins isolated from the insulin producing rat beta cell line, RINmSF showing the effect of interleukin-1β on 12-LO protein expression. In these studies, 25 μg of protein was isolated from the cells grown for 16 hours in reduced serum-containing medium 0.4% alone or along with IL-1β (0.1 ng/ml). The results demonstrate a 2-fold increase in 12-LO protein expression after IL-1 treatment (lanes **2** and **4**). Lane **7** shows the 12-LO standard showing the characteristic 72 kD 12-LO protein band using applicants peptide antibody which recognizes the rat, porcine and human forms of leukocyte-type 12-LO.

FIG. **13** illustrates the effects of IL-1β on 12-HETE production in rat islets.

Applicants have discovered that direct addition of the 12-LO products 12-S-HETE or 12-HPETE directly decreases cellular growth as reflected by decreased ^{3}H thymidine incorporation or DNA synthesis in the rat pure beta cell line RIN-M5F. As shown in FIG. **11**, concentrations of 12-LO products even as low as 10^{-9}M decrease DNA synthesis. FIG. **12** demonstrates that human IL-1B at 0.2 ng increases leukocyte 12-LO protein expression approximately two fold in these RIN cells. FIG. **13** shows that IL-1B at 2.5 ng/ml markedly increases 12-LO activation in freshly isolated rat pancreatic islets as reflected by an increase in immunoreactive release of 12-S-HETE (92 pg/ml/40 islets basal to 250 pg/ml/40 islets.

Role of the 12-LO Pathway in the Development of Autoimmune Inflammatory and Atherosclerotic Disorders in Humans

Increasing evidence suggests that cytokines such as IL_1, IL_4 and IL_8 play a role in the development of autoimmune, inflammatory and atherosclerotic disorders in humans.

DESCRIPTION OF FIGS. 14–20

FIG. **14A** illustrates the dose-dependent effect of the cytokine interleukin-1β on 12-LO mRNA expression in porcine aortic smooth muscle cells (PVSMC). Confluent PVSMC growth in normal glucose medium was serum depleted for 24 hours by placing in medium+0.2% BSA+ 0.4% FCS. This medium was then freshly replaced along with IL-1β and the cells incubated for a further 24 hours. At the end of the incubation, total RNA was extracted from the cells using RNA-STAT. This RNA was the subjected to reverse-transcriptase polymerase chain reaction (RT-PCR) to detect and quantitate leukocyte-type 12-LO mRNA (333 bp PCR product, upper panel) using our well established techniques. The expression of GAPDH mRNA 284 bp, lower panel) was used as an internal control for PCR and for quantitation. The figure clearly shows that IL-1β treatment

leads to a dose-dependent increase in 12-LO mRNA expression (333 bp PCR product) while there is not much change in the internal control, GAPDH mRNA expression.

FIG. **14**B illustrates the effect of the cytokine IL-4 on 12-LO mRNA expression in PVSMC. The ells were treated for 24 hours with IL-4 and 12-LO mRNA quantitated as described in the legend to FIG. **14**A. It is clearly seen that IL-4 also increases 12-LO mRNA expression similar to IL-1β.

FIG. **14**C illustrates the effect of the cytokine IL-8 on 12-LO mRNA expression in PVSMC. The cells were treated for 24 hours with IL-8 and 12-LO mRNA quantitated as described under legend 14A. The figure shows that IL-8 treatment of PVSMC leads to dose-dependent increase in 12-LO mRNA expression while there is no change in the internal control, GAPDH expression.

FIG. **15** illustrates the same RNA analyzed for the internal marker GAPDH.

FIG. **16** illustrates the effect of IL-4 on leukocyte-type 12-LO protein expression in PVSMC.

FIG. **17** illustrates the effect of IL-8 on leukocyte-type 12-LO protein expression in PVSMC.

FIG. **18** illustrates the effect of IL-4 on 12-LO activity in PSMC.

FIG. **19** illustrates the effect of IL-8 on 12-LO activity in PSMC.

FIG. **20** reflects the upregulation of hl 12-LO by IL-1, IL-4 and IL-8.

In addition to this work in islets, applicants have now demonstrated that IL_1, IL_4 and IL_8 can increase the mRNA expression of leucocyte type 12-LO in porcine and human aortic smooth muscle cells. Furthermore, applicants have evidence that 12-LO protein expression is similarly upregulated by these cytokines in porcine vascular smooth muscle. The cells were cultured in DME (normal glucose) and treated for 24 hours±cytokines in medium containing 0.2% BSA and 0.4% serum. Intracellular 12-LO enzyme activity was measured by HPLC, leukocyte-type 12-LO protein expression by immunoblotting and 12-LO mRNA by a specific reverse transcriptase polymerase chain reaction (RT-PCR). All three cytokines (2.5 ng/ml) caused a marked increase in 12-LO enzyme activity 51, 43 and 36% increase in 12-HETE HPLC peak for IL-1, -4 and -8 respectively). Further, all three cytokines (1–5 ng/ml) each led to a potent dose-dependent increase (2–5 fold) in 12-LO mRNA expression (340 bp PCR product). Treatment with these cytokines (0.5–5 ng/ml) also led to an increase (1.4–2.5-fold) in 12-LO protein expression 72 kD). In addition, all three cytokines (2.5 ng/ml) could induce a significant increase in PVSMC DNA synthesis (1.280.08, 1.67±0.11 and 1.3±0.07 fold increase in ^3H thymidine incorporation with IL-1, -4, and -8 respectively, $p<0.01$).

Human vascular smooth muscle cells (HSMC) were also cultured in DME (normal glucose) and treated for 24 hours with IL_1, IL_4 and IL_8 in medium containing 0.2% BSA and 0.4% serum. Intracellular 12-LO enzyme activity in cell sonicates was measured by HPLC, leukocyte-type 12-LO protein expression by immunoblotting and 12-LO mRNA by a specific reverse transcriptase polymerase chain reaction (RT-PCR). Treatment of HSMC in low serum medium for 24 hours with IL-1, IL-4 or IL-8 (5 ng/ml) resulted in 7–10 fold increases in 12-LO mRNA expression relative to untreated cells. RNA from the same experiments was also analyzed for human 12-LO expression by a specific RT-PCR. No 15-LO mRNA was seen either in the basal or after cytokine addition.

These results suggest that these inflammatory cytokines have mitogenic effects in VSMC and that they are potent positive regulators of the 12-lipoxygenase pathway. Thus enhanced 12-LO activity and expression in response to these cytokines may be a key mechanism for cytokine-induced VSMC migration and proliferation observed in atherosclerosis. FIG. **14** shows the effects of IL_1, IL_4 and IL_8 on 12-LO mRNA expression in PVSMC cultured in normal (5.5 mM) and elevated (25 mM) glucoside. FIG. **15** represents the same RNA analyzed for the internal marker gene GAPDH showing all lanes have similar amounts of internal standard RNA. FIGS. **16** and **17** show increases in 12-LO protein expression in PVSMC by IL_4 and IL_8 respectively and FIGS. **18** and **19** reveal HPLC tracings showing selective increases in 12-LO protein activity in PSMC treated with IL_4 and IL_8 respectively. FIG. **20** shows evidence that IL_1, IL_4 and IL_8 can markedly upregulate leucocyte type 12-LO in human aortic SMC. In these same experiments, applicants were unable to demonstrate 15-LO expression in untreated or cytokine treated HSMC demonstrating the selective role of 12-LO as a potential mediator of cytokine action in vascular smooth muscle.

Role of 12-LO Pathway in the Pathogenesis of Type I and II Diabetes

Insulin dependent diabetes or type I diabetes is an autoimmune disease resulting in complete destruction of the insulin producing cells or beta cells in the pancreatic islet. Cytokines such as IL-1β are likely to be involved in this autoimmune process.

It has been discovered that IL-1β induces 12-LO protein and mRNA expression in RIN-M5F cells and 12-LO mRNA expression in rat islets. RIN-M5F cells treated for 16 hours with IL-1β (25, 50 and 100 ng/L) showed a dose dependent two-fold increase in expression of a porcine leukocyte form of 12-LO demonstrated by Western blots. A concomitant increase in 12-LO mRNA expression was seen at this time point using a highly sensitive competitive PCR assay. These. transcriptional and translational events were paralleled by increased 12-LO pathway activity measured by radioimmunoassay for 12-HETE. Additionally, an inhibitor of inducible nitric oxide synthase (iNOS), N-monomethyl arginine (NMMA), was unable to prevent the IL-1β induced increase in 12-LO protein expression in RIN M5F cells, supporting the hypothesis that a pathway independent of inducible nitric oxide (NO) is present. Separate experiments using purified Sprague-Dawley rat islets also showed increased expression of 12-LO mRNA and enzyme activity.

In conclusion, 12-LO is a β-cell specific enzyme regulated at the transcriptional and translational level by cytokines like IL-1β.

DESCRIPTION OF FIGS. 21–24

FIG. **21** reflects the increase in 12-LO mRNA expression in the pancreatic islets of increasingly diabetic rats.

FIG. **22** indicates that 12-LO mRNA expression in diabetic ZDF rat skeletal muscle is higher than from non-diabetic ZDF rats. The ZDF rat model has been proposed as an excellent animal model of spontaneous NIDDM (non-insulin dependent diabetes mellitus).

FIG. **23** presents data pertaining to rat-fibroblasts which overexpress the human insulin receptor.

FIG. **24** reflects a major change in the $HETE/PGI_2$ ratio in various diabetic groups.

NIDDM is a complex genetic disorder associated with a reduced ability of insulin to induce glucose transport in

muscle ("insulin resistance") and a relative impairment of glucose-induced insulin secretion in pancreatic islets.

Consistent with another aspect of this invention, increased activity or expression of the 12-LO pathway is recognized as a common mediator of both of these abnormalities, such that blockade of the 12-LO pathway may prevent development of NIDDM.

The rationale for this statement includes:

1. Highly relevant data which evidences the presence of the leukocyte type 12-LO in pancreatic islets and skeletal muscle. The data in skeletal muscle is new and highly relevant. The available data in rat skeletal muscle and in human islet muscle RNA shows 12-LO expression using PCR analysis.

2. Evidence that 12-LO mRNA expression progressively increases in rat pancreatic islets from lean non-diabetic animals, to obese pre-diabetic and obese diabetic animals (see FIG. **21**).

3. Data that 12-LO products added exogenously to rat pancreatic islets can reduce glucose-induced insulin secretion (**45**).

4. In vivo data (see Table 2) that urinary 12-HETE levels are much higher in male diabetic obese ZDF rats (a model of NIDDM) compared to lean ZDF non-diabetic rats. Interestingly, obese female ZDF rats which are phenotypically like pre-diabetic humans show intermediate levels of 12-HETE in urine.

TABLE 2

Urinary 12-HETE in ZDF Rats

	pg/total urine vol.
Diabetic Male Obese Ctrl	2022 ± 372
Non-diabetic	
Female Obese Ctrl	1007
Female Obese Mg2+	86
Non-diabetic	
Lean Male	—
Lean Female	—

5. Data which indicates that 12-LO mRNA expression in diabetic ZDF rat skeletal muscle is much higher than levels in skeletal muscle from non-diabetic ZDF rats (lane **11** vs lane **21** in FIG. **22**). Interestingly, 12-LO mRNA levels in skeletal muscle are also higher in obese female ZDF rats that are prone to get diabetes. In this figure, the 312 bp band is the 12-LO band while the 281 bp band is the 12-LO competitor. This data represents true competitive PCR analysis of 12-LO mRNA. Applicants also have data in the ZDF heart that suggests that 12-LO expression is also higher in diabetic cardiac tissue.

In addition to this work, 12-LO RNA and protein expression in two NIDDM models, ZDF (Zucker diabetic fatty) rats and GK (Goto Kyoto) rats have been evaluated. A specific quantitative polymerase chain reaction (PCR) assay was used to measure 12-LO mRNA expression and Western blotting using an anti 12-LO peptide antibody was used to evaluate 12-LO protein expression. The GK rat model of NIDDM demonstrated an increased blood glucose concentration compared to age-marched Wistar controls (8.7±0.7 vs 4.8±0.2 mM, $p<0.01$). However, plasma insulin and body weight were similar between the GK and Wistar rats. 12-LO mRNA expression was 4-fold greater in heart from GK rats compared to Wistar ($0.48\pm0.1\times10^5$ molecules per μg RNA in Wistar vs $1.9\pm0.4\times10^5$ in GK $p<0.02$). 12-LO mRNA expression in soleus muscle was over 5-fold greater in GK vs Wistar rats (0.6±0.1 Wistar vs 3.1±0.76 GK). The ZDF obese rats demonstrated an increase in blood glucose concentration and weight compared to the ZDF lean controls (558±75 vs 170±5 mg/dl and 390±7 vs 343±7 gram respectively). 12-LO mRNA was analyzed in the heart, red and white quadriceps muscle in the diabetic and lean ZDF rats. 12-LO mRNA expression was increased by 4–7 fold in the diabetic obese ZDF rats compared to the lean ZDF controls. 12-LO protein expression was similarly increased in heart tissue (5-fold) in the diabetic ZDF vs the lean ZDF controls. These data reflect that muscle 12-LO expression is markedly increased in both lean and obese rat models of NIDDM.

6. Data which indicates that 12-HETE levels in L_6 muscle cells are increased in the presence of glucose. L_6 muscle cells are skeletal muscle cells from rats which have been used to investigate the mechanisms of insulin action. When L_6 muscle cells are incubated in xylose (5.5, mM), they are much more responsive to insulin when compared to cells in regular glucose (5.5 mM) or high glucose (25 mM). Table L below demonstrates higher levels of 12-HETE release in the media (pg/ml) or in the cells (pg/total cells) in regular glucose (Rg) or high glucose (HG) conditions. Thus, elevated 12-Lipoxygenase products, such as 12-HETE, appear to play an important role in reduced insulin metabolic actions caused by high glucose.

TABLE 3

Effect of Glucose on 12-HETE in L_6 Muscle Cells

Cells	Condition		
L6	Xylose	Rg	Hg
pg/ml (n = 2)	53.2	101.8	110.3
pg/total cells (n = 2)	398	472	620

7. Baicalein, a selective 12-LO inhibitor, can prevent glucose-induced insulin resistance. To perform these studies, applicants cultured rat-1-fibroblasts that have been engineered to contain the human insulin receptor for ten days in high glucose (25 mM). For the last 24 hours, the cells were cultured with 10^{-6}M baicalein. The marked bands represent the phosphorylated beta sub-unit of the human insulin receptor (FIG. **23**). As the band becomes lighter, it represents reduced insulin action. Lane **1** represents the insulin receptor phosphorylation in normal glucose (5.5 mM) vs the reduced insulin action in lane **5** (25 mM glucose). Lane **6** represents the insulin receptor phosphorylation in 25 mM glucose when 12-LO pathway was blocked with baicalein showing restoration of insulin receptor phosphorylation.

In addition to this work, applicants have discovered that 12-HETE directly inhibits insulin-induced receptor phosphorylation. As shown in FIG. **27**, a clear increase in phosphorylation of the 97 kD beta subunit of the insulin receptor occurs when insulin is present. This is demonstrated by a darker band at 97 kD in lane **4** (insulin-treated) vs Lane **1** (control, non-insulin-treated). 12-HETE (10^{-7}M concentration) did not directly alter basal insulin receptor phosphorylation (lane **2**). In contrast, 12-HETE markedly reduced insulin-induced receptor phosphorylation of the beta subunit of the human insulin receptor. This is demonstrated by a reduced band intensity in lane **5** (12-HETE 10^{-7}M) vs. Lane **4** (no 12-HETE addition).

Since insulin receptor phosphorylation is one of the early important steps in insulin action, these results suggest that products of the 12-LO pathway can lead to reduced insulin action. This data suggests that increased expression or activity of the 12-LO pathway appears to play a key role in the insulin resistance in non-insulin dependent forms of diabetes.

8. Data which indicates that glucosamine, a proposed major mediator of glucose toxicity in terms of reduced insulin action and vascular disease, increases 12-LO product formation in smooth muscle cells. The role of glucosamine in leading to insulin resistance has recently been demonstrated in intact animals (Baron et al., *J. Clin. Invest.*, 96:2792–2801 (1995)) and in insulin responsive tissues in vitro (e.g., muscle—Robinson et al., *Diabetes,* 42:1333–46 (1993); and fat—Marshall et al., *J. Biol. Chem.*, 266:4706–4712 (1991). Furthermore, glucosamine is thought to be a major mediator of glucose-induced vascular disease (Daniels et al., *Mol. Endocrinol.,* 7:1041–1048 (1993)). While not wishing to be bound by a particular theory, it is believed that glucosamine impairs insulin-induced glucose uptake by blocking the normal action of Glut 4, the major glucose transporter linked to insulin ability to transport glucose.

Porcine vascular smooth muscles cells (PVSMC) were cultured in the presence of glucosamine (7.5 mM) for 24 hours. New media was then added with glucosamine (7.5 mM) for 25 minutes. The cells and media above the cells were collected and 12-HETE was measured using RIA. A very large increase in 12-HETE release into the media in cells cultured in glucosamine compared to those in normal glucose (6.48±1.2 pg/ml 12-HETE release in normal glucose vs. 20.6±3.6 pg/ml released in glucosamine n=4) was observed. Furthermore, cell associated 12-HETE was higher in glucosamine treated cells (735.9±67 pg/cell incubate normal glucose vs. 1225±112 pg in glucosamine). These data suggest that 12-LO products such as 12-HETE may be important factors leading to insulin resistance.

9. Data which indicates that high fat feeding simultaneously leads to impaired insulin action and induction of 12-LO protein expression in muscle. The mouse model used was a transgenic mouse over expressing the Glut 4 transporter (Pfizer Pharmaceuticals). As can be seen in FIG. **28**, high fat feeding clearly led to impaired glucose tolerance, which is a clear indication that the animals were insulin resistant. FIG. **28***a* shows a higher glucose level at every point on the oral glucose test curve in the fat fed mice than in the control. The bar graph of FIG. **28***b* demonstrates a significantly greater area under the glucose tolerance curve in the high fat fed group than in the control.

In four of the animals on the high fat diet, 12-LO protein expression in cardiac muscle was evaluated and compared to the levels in animals on the control diet. 12-LO protein expression was measured using 12-LO peptide antibody and Western blotting. The summary of the data is shown in Table 4 below. The striking results show much higher levels of 12-LO protein (using densitometric analysis of blots) in animals on the high fat diet. These in vivo results suggest that increased 12-LO expression or activity plays a key role in leading to insulin resistance.

TABLE 4

Densitometry Result Comparisons of 12-LO Protein Expression in Heart Muscle Western Blot

Mann-Whitney Test		
Mann-Whitney U-statistic	=	0.000
U'	=	16.000
Sum of ranks in Control Male	=	10.000
Sum of ranks in Fat Male	=	26.000

The two-tailed P value is 0.0286, considered significant.

	Control Diet	High Fat Fed Diet
Parameter:	Control Male	Fat Male
Mean:	2.925	12.897
# of points:	4	4
Std deviation:	1.041	14.636
Std error:	0.5203	7.318
Minimum:	1.890	5.390
Maximum:	4.370	34.850
Median:	2.720	5.675
Lower 95% CI:	1.269	-10.388
Upper 95% CI:	4.581	36.183

10. Data which indicates that high magnesium (Mg) feeding markedly reduces 12-LO gene expression and 12-HETE levels. Mg deficiency has been associated with experimental and human insulin resistance. Moreover, increased dietary Mg has been associated with reduced development of diabetes in ZDF rats as well as in humans, and Mg supplementation can improve insulin response and actions in humans with NIDDM.

It has now been discovered that high Mg feeding markedly reduces 12-LO gene expression and 12-HETE levels. High Mg diets (Purina 5008 diet containing 1% Mg) were fed to one group of ZDF obese (diabetic fatty) male rats while control diets (Purina 5008 plus 0.2% Mg) were fed to another group. As can be seen in Table 5, the high Mg feeding group possessed significantly lower urinary 12-HETE concentrations as measured by RIA (methods described in *J. Clin. Endocrin. Metab.,* 67:584–591 (1988) and *J. Clin. Invest.,* 80:1763–1769 (1987)) than the control group. High Mg feeding also reduced 12-LO mRNA expression in muscle from diabetic ZDF rats.

TABLE 5

Urinary 12-HETE Excretion Rate in ZDF Rat Models

ZDF RAT	12-HETE (pg/min)
ZDF lean (n = 4)	[a]0.4 ± 0.07
ZDF obese (n = 6)	6.12 ± 1.2
ZDF obese (n = 6) with h.Mg diet	[b]3.72 ± 0.5

Values are mean ± SE. n is the number of rats. Values in ZDF lean group and ZDF obese with high magnesium diet group are different from ZDF obese group at $^{a}p < 0.001$ and $^{b}p < 0.05$ respectively.

11. Human data shows increased urinary levels of 12-HETE in people with NIDDM. Levels of 12-HETE are particularly high in diabetics showing evidence of proteinuria. These results suggest that 12-LO activation may be involved in renal disease in diabetes.

Vascular tissue from diabetic animals and man metabolize arachidonic acid differently from normals. PGI_2 is a vasodilator, antithrombolic, and renin secretagogue while 12-hydroxyeicosatetraenoic acid (12-HETE) is proinflammatory and inhibits cyclooxygenase (CO). Applicants earlier

reported a prostacyclin (PGI_2) deficiency in diabetics with hyporeinemic hypoaldosteronism (HH) (**46**). Applicants explored the production of the CO product, PGI_2 and the lipoxygenase (LO) product, 12-HETE in NIDDM patients with normal renal function (NR), those with microalbuminuria (MiAO, macroabluminuria (MaA) and HH patients. PGI_2 (6 keto PGF^1) and 12-HETE were measured in urine by HPLC followed by RIA using published methods. Results are:

	PGI_2 (ng/gm Creat)	12-HETE (ng/gm Creat)	Ratio
Controls (N = 17)	64 ± 16	43 ± 9	0.7 ± 0.3
Diabetics (NR) (N = 8)	64 ± 9	122 ± 34*	2.0 ± 0.5
Diabetics (MiA) (N = 14)	75 ± 10	226 ± 60*	3.8 ± 1.3
Diabetics (MaA (N = 9)	48 ± 7**	352 ± 152*	8.1 ± 5.4
Diabetics (HH) (N = 5)	39 ± 5	240 ± 35*‡	6.8 ± 2.4

‡From previously stored samples
*$p < 0.01$ vs controls
**$p < 0.05$ vs diabetics

This data suggests that (1) an increase in the 12-LO product 12-HETE is observed in all NIDDM which progresses with renal disease; (2) diabetic renal disease with albuminuria is associated with suppression of PGI_2 production; and (3) HH is a disorder of PGI_2 suppression and 12-HETE excess.

Applicants further study has measured urinary (renal vascular) production of both PGI_2 and 12-HETE in patients with varying degrees of diabetic renal involvement.

In the group of NIDDM patients with normal renal function based on creatinine clearance and urinary albumin measurements, PGI_2 excretion was not different from normal controls. The group with microalbuminuria were divided into those with hypertension and normotensive. However, no difference in PGI_2 excretion was noted. Nevertheless the microalbuminuria group had significantly lower PGI_2 excretion rates. The patients with macroalbuminuria and reduced creatinine clearance similarly had reduced PGI_2 excretion. PGI_2 excretion rates were reduced in the macroabluminuria and HH group.

12-HETE values were markedly increased in NIDDM patients with or without microalbuminuria compared with normal controls. 12-HETE excretion values were also significantly increased in the macroabluminuria group as well as in the HH group.

Low dose calcium infusions have been previously shown to increase PGI_2, probably via activation of tissue phospholipases. When a three hour infusion of calcium gluconate was administered to normal subjects, there was a highly significant increase in both PGI_2 and 12-HETE. However, when administered to NIDDM patients with microalbuminuria, there was no increase in PGI_2 but a further stimulation of the already increased 12-HETE values. This supports the concept that a defect in prostacyclin formation exists in NIDDM.

12-HETE/PGI_2 ratios were calculated as an additional approach to define whether in NIDDM there is an alteration in the LO/CO pathways. As shown in FIG. **24**, there is a major change in the HETE/PGI_2 ratio in all diabetic groups. The mean value is significantly increased in NIDDM patients with normal renal function and is further altered in patients with macroalbuminuria and HH patients. However, these differences were not significantly different between the NIDDM groups due to the variability within each group.

Applicants data appears to exclude renal function per se, GFR or hypertension as a cause of the deranged eicosanoid excretion values. While the origin of PGI_2 and 12-HETE in urine has not been fully settled, studies using extrarenal Co inhibitors and lack of excretion of tracer PGI_2 and 12/15-HETE following systemic injection, suggests hat the kidney is the major source of these compounds in urine (**47–49**). HETEs can be generated in vascular tissue as well as from inflammatory cells. (**50–51**). However, there is no evidence for macrophage/leukocytes infiltration into the kidney in NIDDM with only incipient glomerular and vascular disease.

In agreement with in vitro and animal model studies cited earlier, applicants results suggest that early in diabetes mellitus, there is fixed prostacyclin production which falls to lower values with diabetic renal vascular/glomerular disease. This occurs in a state where the LO product 12-HETE is increased early in diabetes mellitus prior to development of microalbuminuria. These observations could be of considerable importance in the etiology of diabetic vascular disease since the HETEs are mitogenic proinflammatory, vasoconstrictive, and stimulate angiogenesis (**52–53**). With respect to the HH syndrome, applicants new data suggests that increased HETE production may be an early abnormality in suppressing PGI_2 formation and renin biosynthesis and secretion. The etiologic event in diabetes explaining vascular disease is not known. However, recent studies suggest that hyperglycemia per se has a number of metabolic consequences including enhanced eicosanoid generation via protein kinase C and activation of calcium dependent phospholipases, major mediators of AA release (**54**).

In summary, whether cause or effect, very early involvement of the kidney in diabetes is associated with fixed or suppressed production of prostacyclin, with increase in the vasculotoxic lipoxygenase product 12-HETE. This conclusion is now suggested by both in vitro studies and in vivo studies in man. This suggests pharmacologic intervention early in the diabetic state to block this derangement.

Role of 12-Lipoxygenase Products in Glucose-Induced Monocyte Binding to Human Aortic Endothelial Cells

The rate of atherosclerosis is accelerated in humans with diabetes mellitus (DM). Applicants recently published evidence that high glucose (HG) exposure of human aortic endothelial cells (HAEC) selectively increases monocyte (MO) but not neutrophil binding (**56**). HG exposure to EC can increase arachidonic acid (AA) release and lipoxygenase (LO) production formation. In the current study, applicants evaluated the role of 12- and 15-LO products in MO binding to HAEC. Culture of HAEC in HG (25 mM) for two passages increased MO binding compared to cells maintained in 5.5 mM glucose (239±30 cells/field HG vs 111±7, $p<0.01$). Phenidone (10^{-6}M), an inhibitor of the 12-LO pathway (50 percent inhibition of HG-induced binding, $p<0.05$). HG culture of HAEC significantly increased both 12- and 15-hydroxyeicosatrienoic acids (HETEs) using applicants HPLC and RIA methods. 12(S)-HETE added to HAEC cultured in 5.5 mM glucose increased MO binding (66±6 cells/field control vs 114±4 12-HETE 10^{-10}M, $p<0.01$). Another novel 12-LO product 12(R)-Hydroxyeicosatrienoic acid was even more potent showing effects on MO binding at 10–11 and 10^{-12}M. In contrast, 15(S)-HETE added at concentrations ranging from 10^{-6} to 10^{-12}M did not stimulate MO binding to HAEC. In summary, (1) elevated glucose increases MO binding to HAEC and this effect can be reduced by blockade of the LO

pathway (2) 12 but not 15-LO products, can increase MO binding. Since a leucocyte type 12-LO is expressed in HAEC, these results support the role of 12-LO activation in glucose-induced MO binding to human endothelium.

Applicants have also demonstrated that high fat "cafeteria" diets increase leukocyte 12-LO in rat hearts and that diabetic (GK) rats have a higher 12-LO in heart compared to normal (Wistar) animals (see FIGS. **25** and **26**).

DIAGNOSTIC ASSAYS

Application PCT/US94/00089 reports that antibodies would circulate in patients at risk for developing disease states for which hl 12-LO or its pathway products such as HETE or 12-HPETE are the etiological agent. Accordingly, another aspect of this invention includes assays, e.g., of the ELISA type in which hl 12-LO protein, or a related material such as HETE is utilized as an immunogen. Such tests are useful to diagnose any of the disease states mediated by the activation or expression of hl 12-LO.

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What is claimed is:

1. A method for inhibiting the etiology of disease in a human patient suffering from or at risk for a disease state in which hl 12-lipoxygenase or 12-HETE is an etiological agent which comprises administering to said human patient a hl 12-lipoxygenase pathway inhibitor in an amount effective to retard or inhibit the expression or activity of said hl 12-lipoxygenase; provided that said hl 12-lipoxygenase pathway inhibitor is not aminoguanidine or pioglitazone.

2. The method of claim 1, wherein said disease state is Type II diabetes or breast cancer.

3. The method of claim 1, wherein said disease state is non-insulin dependent diabetes mellitus and said hl 12-lipoxygenase pathway inhibitor is administered in an amount therapeutically effective to enhance the ability of insulin to induce glucose transport in muscle and the secretion of insulin in pancreatic islets.

4. The method of claim 1, in which said hl 12-lipoxygenase pathway inhibitor is NDGA, CDC, panaxynol, baicalein or a ribozyme which cleaves hl 12-lipoxygenase mRNA.

5. A method for inhibiting the proliferation of breast cancer tissue in a human patient which comprises administering to said patient a therapeutically effective amount of a drug which inhibits hl 12-lipoxygenase expression or activation; provided that said hl 12-lipoxygenase inhibitor is not aminoguanidine or pioglitazone.

6. The method of claim 5, in which said hl 12-lipoxygenase inhibitor is NGDA, CDC, panaxynol, baicalein, or a ribozyme which cleaves hl 12-lipoxygenase mRNA.

7. The method of claim 5, in which the proliferation of breast cancer tissue is basal, epidermal growth factor-induced or estrogen-induced.

8. A method for mediating breast cancer cell growth and development which comprises administering to a patient in need thereof a therapeutically effective amount of a hl 12-lipoxygenase pathway inhibitor; provided that said hl 12-lipoxygenase pathway inhibitor is not aminoguanidine or pioglitazone.

9. The method of claim 8, in which said hl 12-lipoxygenase pathway inhibitor is NGDA, CDC, panaxynol, baicalein, or a ribozyme which cleaves hl 12-lipoxygenase mRNA.

10. The method of claim 8 in which the breast cancer cell growth and development is basal, epidermal growth factor-induced or estrogen-induced.

11. A method for inhibiting the etiology of disease in a patient having a disease state in which 12-HETE is an etiological agent which comprises administering a hl 12-lipoxygenase pathway inhibitor which decreases mitogenic activity in said patient; provided that said hl 12-lipoxygenase pathway inhibitor is not aminoguanidine or pioglitazone.

12. The method of claim 11, wherein said hl 12-lipoxygenase pathway inhibitor reduces ERK, JAK, PAK, and/or JNK activity in said patient.

13. The method of claim 12, wherein said hl 12-lipoxyenase pathway inhibitor reduces JNK activity by decreasing PAK activity.

14. The method of claim 11, wherein the disease state is Type II diabetes or breast cancer.

15. A method for increasing insulin receptor phosphorylation in a patient having Type II diabetes which comprises administering to the patient a therapeutically effective amount of a hl 12-lipoxygenase pathway inhibitor sufficient to inhibit hl 12-lipoxygenase pathway products from inhibiting insulin receptor phosphorylation; provided that said hl 12-lipoxygenase pathway inhibitor is not aminoguanidine or pioglitazone.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 6,191,169 B1
DATED : February 20, 2001
INVENTOR(S) : Jerry Nadler et al.

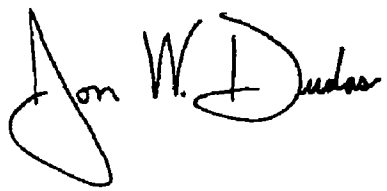
It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 10,
Line 64, "HC" should read -- HAEC --.

Column 26,
Line 63, "10-11" should read -- 10^{-11} --.

Signed and Sealed this

Twenty-seventh Day of July, 2004



JON W. DUDAS
Acting Director of the United States Patent and Trademark Office

Exhibit 6

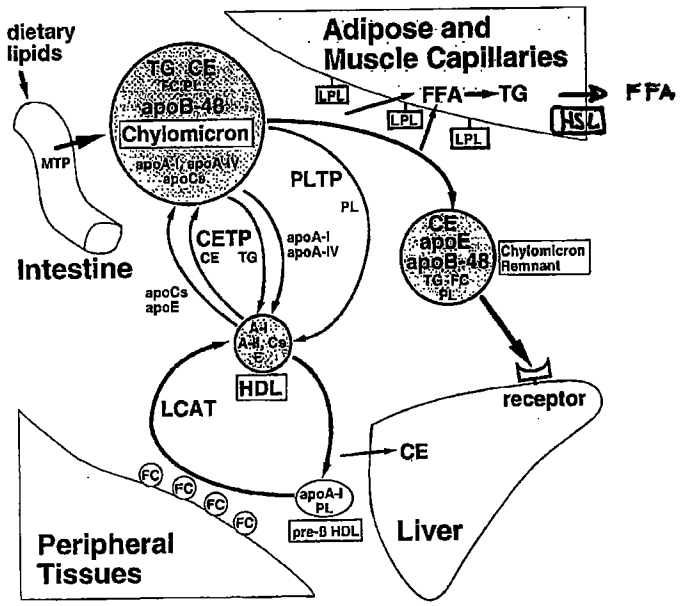


Figure 1. Exogenous lipid transport: FC, free cholesterol; CE, cholesteryl ester; PL, phospholipid; TG, triglyceride; FFA, free fatty acid; LPL, lipoprotein lipase; LCAT, lecithin–cholesterol acyltransferase; CETP, cholesteryl ester transfer protein; PLTP, phospholipid transfer protein; MTP, microsomal triglyceride transfer protein.

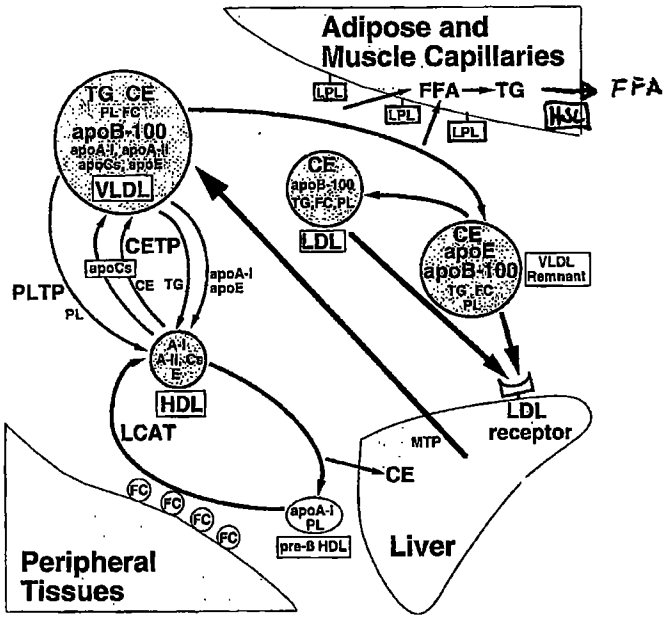
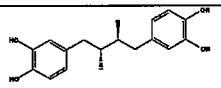
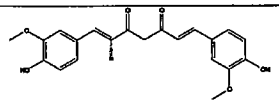
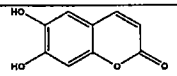
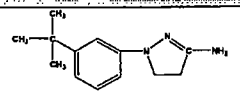
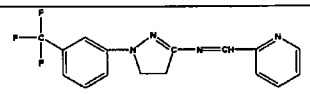
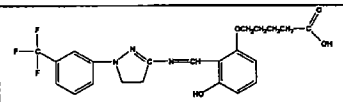
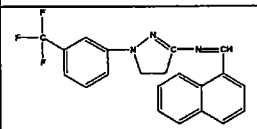
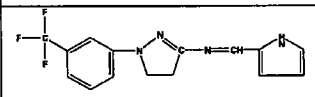
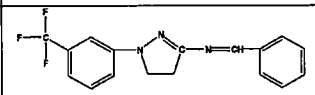
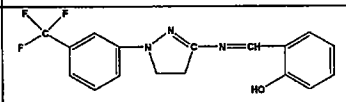
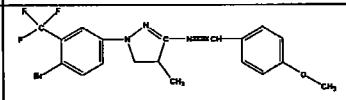
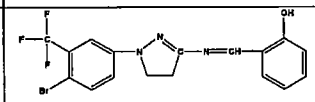
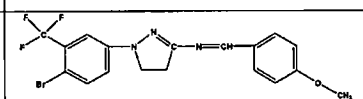
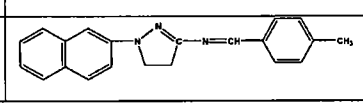


Figure 2. Endogenous lipid transport: FC, free cholesterol; CE, cholesteryl ester; PL, phospholipid; TG, triglyceride; FFA, free fatty acid; LPL, lipoprotein lipase; LCAT, lecithin–cholesterol acyltransferase; CETP, cholesteryl ester transfer protein; PLTP, phospholipid transfer protein; MTP, microsomal triglyceride transfer protein.

Exhibit 7

Structure Comparison of Lipoxygenase Inhibitors

Compound	Structure	Lipoxygenase inhibitory activity	Triglyceride lowering
Masoprocol, nordihydroguiaiaretic acid (NDGA) Triglyceride lowering effect is excreted through the hormone sensitive lipase inactivation (*see* specification [0004])		**YES**	**YES**
Curcumin The molecular aspects of the triglyceride lowering activity have not been determined (*see* specification [0006])		**YES**	**YES**
Esculetin (Gowri *et al.*, 2000)		**YES**	**NO**
4,5,dihydro-1-(3-trifluoromethylphenyl)-1H-pyrazol-3-amine (*see* specification table 2)		**YES**	**YES**
3-Amino-1-(3-t-butylphenyl)-2-pyrazoline (US 4,572,912)		$IC_{50}(\mu M) = <1$	ND
3-(2-pyridylmethyleneamino)-1-(3-trifluoromethylphenyl)-2-pyrazoline (US 4,572,912)		$IC_{50}(\mu M) = \sim 3$	ND
3-(2,4'-carboxybutoxy-6-hydroxybenzylidine-amino)-1-(3-trifluoromethylphenyl)-2-pyrazoline (US 4,572,912)		$IC_{50}(\mu M) = 10\text{-}20$	ND
3-(1-naphthylmethyleneamino)-1-(3-trifluoromethylphenyl)-2-pyrazoline (US 4,572,912)		$IC_{50}(\mu M) = 6$	ND
3-(2-pyrrolylmethyleneamino)-1-(3-trifluoromethylphenyl)-2-pyrazoline (US 4,572,912)		$IC_{50}(\mu M) = 1$	ND
3-Benzylideneamino-1-(3-trifluoromethylphenyl)-2-pyrazoline		$IC_{50}(\mu M) = <1$	ND
3-salicylidenamino-1-(3-trifluoromethylphenyl)-2-pyrazoline (US 4,572,912)		$IC_{50}(\mu M) = \sim 3$	ND
4-Methyl-3-salicylideneamino-1-(3-trifluoromethylphenyl)-2-pyrazoline (US 4,572,912)		$IC_{50}(\mu M) \sim 1$	ND
1-(4-bromo-3-trifluoromethylphenyl)-3-(2-hydroxybenzylideneamino)-2-pyrazoline (US 4,572,912)		$IC_{50}(\mu M) = >10$	ND
1-(4-bromo-3-trifluoromethylphenyl)-3-(4-methoxybenzylideneamino)-2-pyrazoline (US 4,572,912)		$IC_{50}(\mu M) = \sim 1$	ND
3-(4-methylbenzylideneamino)-1-(2-naphthyl)-2-pyrazoline (US 4,572,912)		$IC_{50}(\mu M) = 12$	ND